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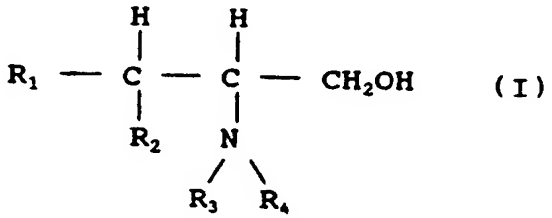

(I)

(57) Abstract

The present invention provides methods and compositions for inducing differentiation of cells. Compositions having formula (I) wherein R_1 is C_1 to about C_{20} alkyl or alkenyl; R_2 is hydroxyl, alkoxy or H, R_3 is H or lower alkyl; R_4 is COR_5, SO_2R_5, or CSR_5, where R_5 is C_1 to C_{20} alkyl, alkenyl, or alkynyl, which may be substituted by one or more of the following functional groups: OH, SH, OR_6, SR_6, NR_7R_8, $COOR_9$, and $CONR_{10}R_8$, where R_6, R_7, R_8, R_9, and R_{10} independently are H, alkyl, aryl, alkaryl and arylalkyl using up to about 10 carbons, are administered to cells of a mammal that are capable of undergoing differentiation in amounts effective to induce differentiation of the cells. The invention also provides methods and compositions for altering the phenotype of cells and for treating diseases characterized by hyperproliferation of cells.

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METHODS FOR INDUCING CELL DIFFERENTIATION USING CERAMIDES

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of co-pending application Serial No. 566,978 filed August 13, 1990 the disclosures of which are hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to the field of compounds and methods for inducing cell differentiation. More particularly the present invention is concerned with the use of ceramide and derivatives to induce cell differentiation and for treatment of conditions characterized by abnormal cell proliferation.

REFERENCE TO GOVERNMENT SUPPORT

The research disclosed herein was supported in part by National Institutes of Health grants ES 00155 and CA 46738. The United States government has certain rights in the invention.

BACKGROUND OF THE INVENTION

A number of human malignant and non-malignant diseases have as one of their distinguishing features the hyperproliferation of cells. In these diseases, cells proliferate at abnormally high rates. The cells found in cancerous tumors and leukemias grow and divide uncontrollably, which accounts in part for their rapid spread in the body. Similarly, with some non-malignant diseases such as psoriasis, the cells also grow and divide at abnormally high rates. In

these diseases, the hyperproliferating cells are present in a relatively undifferentiated state. Undifferentiated cells are able to grow and divide. Once a cell differentiates, however, it loses the ability to proliferate. Some proposed treatments have been aimed towards inducing cell differentiation to stop cell proliferation, and thus bring the diseases under control.

Recently it has been found that sphingolipids play important roles in cell growth, oncogenesis, and differentiation (Hannun, Y.A. and Bell, R. M. (1989) Science 243: 500-507). Sphingolipid breakdown products are emerging as a novel class of cell regulatory molecules. Sphingolipid breakdown products, sphingosine and lysosphingolipids, inhibit protein kinase C, believed to be a pivotal enzyme in cell regulation and signal transduction (Hannun, Y.A. et al. (1986) J. Biol Chem. 261: 12604-12609). Sphingolipids and lyso-sphingolipids affect significant cellular responses and exhibit anti-tumor promoter activities in various mammalian cells (Hannun, Y.A. and Bell, R.M. (1987) Science 235: 670-674; Hannun, Y.A. et al. (1987) J. Biol. Chem. 262: 13620-13626; and Wilson, E. et al. (1987) Arch. Biochem. Biophys. 259: 204-214).

U.S. Patent 4,710,490 issued December 1, 1987 to Catsimpoolas discloses compositions which contain lipid containing molecules possessing angiogenic activity. The lipids are derived from mammalian sources, particularly the omentum. Mixtures of known lipids, such as gangliosides, were also found to possess angiogenic activity. The compositions stimulated the growth of blood vessels in vitro, and in vivo. Gangliosides possessed the greatest angiogenic activity, whereas glycolipids such as ceramide derivatives had little or no activity.

U.S. Patent 4,673,667 issued June 16, 1987 to Catsimpoolas discloses plasmin inhibitory substances derived from mammalian omental extracts. The substances contain lipid components. Gangliosides exhibited the greatest plasmin inhibiting activity. Various ceramide or ceramide derivative

samples exhibited no or minimal plasmin inhibiting activity.

Japanese patent application H1-93562 published April 12, 1989 discloses sphingosine derivatives that are useful for the treatment of tumors.

U.S. patent 4,816,450 issued March 28, 1989 to Bell discloses long chain bases, generally sphingosine and sphingosine derivatives, useful for inhibiting protein kinase C. Activation of protein kinase C has been identified as fundamental to tumor promotion, cellular transformation and to understanding the inhibition by anti-tumor agents.

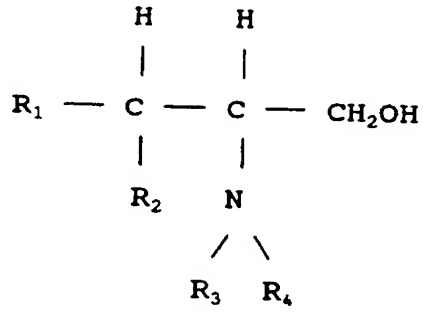
Interferon which induces cell differentiation has been tested for treatment of tumors. Similarly, vitamin D_3 which induces differentiation of HL-60 cells, a human myelocytic leukemia cell line, has also been tested for tumor treatment. Although vitamin D_3 is able to induce cell differentiation, the use of this compound for treating tumors is not feasible since the large amounts of vitamin D_3 needed interferes with calcium metabolism in the body to an unacceptable degree.

Despite the efforts in developing treatments for diseases characterized by cellular hyperproliferation, there is still a need for treatments for these diseases. Accordingly, it is an object of the invention to provide methods and compositions for inducing cell differentiation. It is also an object of the invention to provide methods and compositions for altering the phenotype of cells. It is a further object to provide methods and compositions for treating diseases characterized by hyperproliferation of cells. Yet another object is to provide compositions for prevention and palliation of diseased or abnormal states in mammals characterized by abnormal cell differentiation. Other objects of the invention will become apparent from a review of the present specification and appended claims.

SUMMARY OF THE INVENTION

The present invention provides compositions and methods for inducing cell differentiation. The invention also provides methods and compositions for altering the phenotype

of cells and treating diseases characterized by hyperproliferation of cells. In the methods of the invention, compounds are administered to a mammal, usually a human patient, in therapeutically effective amounts, such compounds having formula I:



I

wherein R_1 is C_1 to about C_{20} alkyl or alkenyl;

R_2 is hydroxyl, alkoxy or H,

R_3 is H or lower alkyl;

R_4 is COR_5, SO_2R_5, or CSR_5, where R_5 is C_1 to C_{20} alkyl, alkenyl, or alkynyl, which may be substituted by one or more of the following functional groups: OH, SH, OR_6, SR_6, NR_7R_8, $COOR_9$, and $CONR_{10}R_8$, where R_6, R_7, R_8, R_9, and R_{10} independently are H, alkyl, aryl, alkaryl and arylalkyl using up to about 10 carbons.

The compounds of the invention are useful in treating conditions where hyperproliferation of cells is present or there is significant disturbance in differentiation of cells. Accordingly, the compounds and pharmaceutical preparations of the invention are useful in the preparation and/or manufacture of a medicament for inducing differentiation of cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a graph of the dose-dependence of ceramide in response to 1,25-$(OH)_2D_3$.

Figure 2 shows a graph of the time course of mass increase of ceramide in response to 1,25-$(OH)_2D_3$.

Figure 3 graphs the mass changes of total phospholipids (A), phosphatidylcholine (B), and sphingomyelin (C) in response to 1,25-$(OH)_2D_3$ treatment of HL-60 cells.

Figure 4 shows the ability of C_{18}/C_2 ceramide and 1nM 1,25-$(OH)_2D_3$ to induce HL-60 cell differentiation.

Figure 5 shows a graph of the effects of C_{18}/C_2 ceramide on HL-60 cell growth.

Figure 6 shows a graph of the effects of C_{18}/C_2 ceramide on HL-60 cell differentiation in the absence of 1,25-$(OH)_2D_3$.

Figure 7 shows a graph of the time course of HL-60 cell differentiation induced by C_{18}/C_2 ceramide.

Figure 8 shows the effects of C_{18}/C_2 ceramide on mass of sphinglmyelin in HL-60 cells.

Figures 9 and 10 show graphs of the effects of transient increase of C_{18}/C_2 ceramide on HL-60 cell differentiation.

Figure 11 shows an autoradiogram of a thin layer chromatography (TLC) plate after 24 hours exposure of the uptake and metabolism of [^{3}H]C_{18}/C_2 ceramide into HL-60 cells.

Figure 12 shows a graph of the lipids extracted from HL-60 cells showing the uptake and metabolism of [^{3}H]C_{18}/C_2 ceramide into HL-60 cells.

Figure 13 shows graphs of the effects of sphingosine on 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Applicants have discovered that administration of ceramide and derivatives of ceramide induces differentiation of HL-60 cells, a line of myelocytic leukemia cells. Administration of ceramide or derivatives of ceramid slows proliferation of the cells and induces the cells to display a differentiated phenotype indicative of normal monocyte cells. It is believed that this effect will be manifested in other types of cells as well.

The human cell line HL-60, originally isolated from a patient with acute myelocytic leukemia, is frequently used to study myeloid cell differentiation. These cells can be induced to mature into granulocytes when treated with agents such as dimethyl sulfoxide or retinoic acid, or into monocyte/macrophage-like cells upon incubation with phorbol

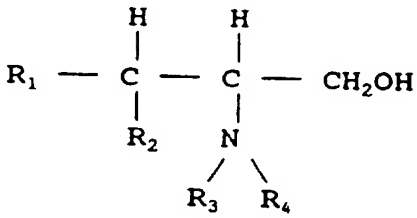
esters, 1α,25-dihydroxyvitamin D_3, or ganglioside G_{M3}. The mechanism by which maturation is caused by most of these compounds is not known. For a review of the characteristics of the HL-60 promyelocytic leukemia cell line and its use as a model for the study cell of differentiation see Collins, S.J. (1987) Blood 70: 1233-1244. This well-known cell model has been used to show the usefulness of the compounds of this invention for treating diseases characterized by cell hyperproliferation.

In prior work (Okazaki, T. et al. (November 15, 1989) J. Biol. Chem. 264: 19076-19080), it was reported that differentiation of HL-60 cells with $1,25\text{-}(OH)_2D_3$ was accompanied by sphingomyelin turnover. It was found that activity of a neutral sphingolyelinase, detected in extracts of HL-60 cells, was induced by $1,25\text{-}(OH)_2D_3$ treatment and was accompanied by the generation of ceramide and phosphoryl-choline. Sphingomyelin, ceramide and phosphorylcholine levels returned to baseline levels within four hours, suggesting a resynthesis phase of sphingomyelin, thus completing a sphingomyelin cycle. These observations are believed to indicate the operation of a sphingomyelin cycle in which inactive parental sphingolipids are converted to active metabolites during cell activation. Unlike the phosphatidylinositol cycle, sphingomyelin turnover occurs over a longer period and may be involved with longer term cell changes.

At least 300 different sphingolipids are synthesized in various mammalian cell types. Structurally, sphingolipids are composed of a long-chain sphingoid base, an amide-linked fatty acid, and a polar head group at the 1-position. Except for ceramide, which has hydroxyl at the 1-position, and for sphingolmelin, which has a phosphorylcholine head group, all other sphingolipids contain carbohydrate head groups and hence are designated glycosphingolipids. These neutral lipids, contain from one (cerebrosides) to 20 or more glucose units, while acidic glycosphingolipids, contain one or more sialic acid residues (gangliosides) or sulfate monoester groups

(sulfatides). Most of the gangliosides and complex glycolipids are thought to reside on the outer leaflet of the cell membrane. Sphingomyelin, however, also resides in the interior of the cell.

The compounds suitable for use in the invention may be naturally occurring or synthetically produced. Compounds having formula I are suitable for use in the pharmaceutical preparations and methods of the invention.



I

wherein R_1 is C_1 to about C_{20} alkyl or alkenyl;
R_2 is hydroxyl, alkoxy or H,
R_3 is H or lower alkyl;
R_4 is COR_5, SO_2R_5, or CSR_5, where R_5 is C_1 to C_{20} alkyl, alkenyl, or alkynyl, which may be substituted by one or more of the following functional groups: OH, SH, OR_6, SR_6, NR_7R_8, $COOR_9$, and $CONR_{10}R_8$, where R_6, R_7, R_8, R_9, and R_{10} independently are H, alkyl, aryl, alkaryl and arylalkyl using up to about 10 carbons.

The compounds are preferably cell soluble, i.e., able to pass through the cell wall and enter the interior of the cell. Compounds wherein R_1 and R_4 taken together have from about 10 to about 28 carbons are more preferred. The total length of the carbon chains of R_1 and R_4 may be divided in any combination between R_1 and R_4 provided that R_1 and R_4 each contain at least one carbon. For instance, R_1 could be C_1 and R_4 could be C_{12}, or R_1 could be C_{10} and R_4 could be C_7. It has been found that compounds of this size are more easily able to pass through the cell membrane and enter the interior of the cell.

R_1 is preferably C_1 to about C_{20} alkyl or alkenyl; more preferably C_1 to about C_{20} alkyl or C_1 to C_{12} alkyl or

alkenyl. R_2 is preferably H, hydroxyl or alkoxy, more preferably hydroxyl or alkoxy. In one preferred embodiment R_2 is methoxy. R_3 is preferably H or lower alkyl (i.e. C_1 to C_6 alkyl), more preferably H.

R_4 is preferably COR_5, SO_2R_5 or CSR_5, where R_5 is C_1 to C_{20} alkyl, alkenyl, or alkynyl which may be substituted by one or more of the functional groups OH, SH, OR_6, SR_6, NR_7R_8, $COOR_9$ and $CONR_{10}R_8$, where R_6, R_7, R_8, R_9 and R_{10} independently are H, lower alkyl (i.e C_1 to C_6) and C_1 to C_{12} aryl, alkaryl, and arylalkyl. More preferably R_4 is COR_5 where R_5 is C_1 to C_{20} alkyl or alkenyl. Suitable aryl include phenyl, substituted phenyl and pyridine. Suitable arylalkyl include benzyl and phenethyl.

Preferred compounds include ceramide, C_{18}/C_2 ceramide, C_{18}/C_6 ceramide, C_{11}/C_8 ceramide and 3-O-methyl-sphingosine. The nomenclature for these compounds is explained in the Experimental section of this specification.

Compounds of this invention are useful for inducing cellular differentiation. They are administered to cells in a mammal, usually a human patient, that are capable of differentiation in an amount effective to induce differentiation of the cells. The terms cellular differentiation, differentiation of cells and similar terms are intended to refer to the biological process wherein cells mature and acquire the characteristics of a functional cell. During differentiation the cell may, for example, acquire or loss morphological shape or characteristics, and gain or lose the ability to bind substances or perform chemical reactions. The term inducing differentiation is intended to refer to the acts of manipulating cells that are capable of differentiation to acquire a differentiated phenotype. Generally, mammalian cells begin as immature, undifferentiated cells that then undergo differentiation during which time they acquire the characteristics of mature, differentiated cells.

Compounds having the structure of formula I are also useful for altering the phenotype of cells. The shape, behavior and other characteristics of a cell including

biochemical activities are generally known as the phenotype of a cell. In this embodiment of the invention, the compounds of the invention are administered to cells having a transformed phenotype in a mammal, usually a human patient, in an amount effective to alter the phenotype of the cell to a phenotype associated with normal cells of the same kind. The "normal" phenotype of a cell refers to a cell that appears normal by conventional criteria such as shape, markers, growth, response to environment, and regulations. Transformed cells are cells that have been derived from normal cells, either spontaneously or by manipulation, that have acquired cancer-like properties such as more immature/undifferentiated phenotype, increased growth, poor or no response to environment and to controls of cell growth, or the ability to cause tumors in animal models. Altering the phenotype of a cell thus refers to the acts of changing at least one characteristic of the cell, including the ability to bind compounds, express enzymatic activity, response to its environment and other cellular characteristics.

Because of the ability of the compounds of this invention to induce cell differentiation and alter the phenotype of cells, such compounds are expected to be useful for treatment of diseases characterized by hyperproliferation of cells, or where there is significant disturbance in differentiation of cells. Diseases characterized by hyperproliferation of cells include diseases wherein one of the consequences or manifestations of the disease is abnormal proliferation of the involved cells.

Abnormal proliferation of cells is generally manifested by an increase in the number of cells present when compared to the number of cells present in the absence of disease. Hyperproliferation of cells may occur in normal, abnormal or malignant cells. Diseases that may be characterized by hyperproliferation of cells include cancerous tumors, leukemias, non-malignant tumors, psoriasis, atherosclerosis and other diseases. This list is intended to be illustrative and not exhaustive of such diseases. These

diseases share in the fact that they are primarily caused by increased and abnormal proliferation of either malignant (e.g. cancer, leukemia and lymphoma), premalignant (e.g. myelodysplasia), or benign (e.g. lymphoproliferative, benign tumors, and psoriasis) cells. Inhibition of cell proliferation by compounds in accordance with this invention may slow the growth of affected cells in these diseases yielding a significant therapeutic and potentially curative effect.

Many disorders of this type are also characterized by having undifferentiated cells. Undifferentiated cells or undifferentiated phenotype refers to immature cells that are usually unable to function as mature cells because they lack the necessary biochemical and physiological machinery characteristic of mature cells. During the process of cell differentiation, immature cells begin to express the biochemical and physiological characteristics of mature cells. For example, *in vivo*, stem cells differentiated into granulocytes, and monocytes. The inability of undifferentiated cells to change into more differentiated cells having a healthier phenotype, contributes to the lack of normal function. The ability of compounds in accordance with this invention to induce differentiation should also help in attenuating the increased proliferation of these cells and in allowing the cells to acquire the necessary biochemical and phenotypic characteristics that allow them to function as normal cells. For example, in the case of psoriasis, the compounds of of this invention are believed to induce differentiation of the abnormal proliferating cells in psoriasis which should allow the cells to differentiate into healthy skin. Similarly, in milodysplasias, these compounds are believed to cause differentiation of early and undifferentiated myeloid cells which may play a significant role in combating the main health hazards from these disorders, i.e. the decreased numbers of normal, well-differentiated blood cells. Leukemia, lymphoma, and other forms of cancer may also be treated by increasing the differentiation of those malignant cells. Since

differentiated cells are usually unable to divide, this helps in treating those diseases since the individual cells will no longer be able to replenish the malignant clone and will not be able to metastasize.

The utility of the compounds in accordance with this invention versus a wide array of neoplastic disease is also strongly supported by the observations that both tumor necrosis factor (TNF) and gamma interferon elevate the levels of ceramide and that ceramide may mediate the effects of these agents on HL60 cell differentiation. Therefore, these compounds are expected to be useful in cancer treatment by inducing tumor necrosis and tumor regression.

Since ceramide and derivatives are able to slow the growth of lymphocytes, the compounds of this invention are also believed to be useful in inducing immunosuppression in mammals, particularly humans. Other agents that suppress the growth of lymphocytes such as steroids, anti-lymphocyte antibodies, and others play important roles in inducing immunosuppression. Immunosuppression is very important in organ rejection such as occurs in renal transplant, heart transplant, liver transplant and other organ transplant. Also, since steroids increase ceramide, ceramide may mimic the effects of steroids as immunosuppressants. The slowing of the growth of cells, particularly lymphocytes, refers to retarding or inhibiting the normal rate of growth and cell division of the cells. Thus compounds that slow the growth of cells have the effect of slowing the rate of growth and normal function of those cells.

Auto-immune disorders are characterized by increased activity and proliferation of self-reactive lymphocytes. The ability of ceramide to potentially suppress growth of lymphocytes is expected to significantly contribute to suppressing manifestations of autoimmune disorders. Since corticosteroids increase the levels of ceramide, and corticosteroids have a therapeutic role in autoimmune disorders, it is now believed that ceramide and other compounds in accordance with this invention may mediate the

action of steroids in these disorders.

Obesity may also be characterized by increased proliferation and metabolism of fat cells in the body. The compounds of this invention may slow the growth of these cells and thus contribute to the reduction of obesity. A major connection arises from the fact that tumor necrosis factor increases the levels of ceramide. TNF is postulated to play a role in inducing cachexia and has been implicated as a potential therapy for obesity. It is now believed that ceramide may be useful for treatment of obesity.

Atherosclerosis is another disease characterized by increased and possibly abnormal proliferation of smooth muscle cells and endothelial cells. It is expected that slowing the growth of these cells by treatment with the compounds of this invention will contribute to the control of atherosclerosis.

The compounds of this invention are also expected to be useful as an anti-skin aging treatment. Retinoic acid, which is known for use in anti-skin aging treatments, elevates the levels of ceramide in skin cells. Since retinoic acid elevates the levels of ceramide, ceramide may mediate the action of retinoic acid and be useful in anti-aging skin treatments.

The present invention may also be useful in chemo-prevention, i.e. the treatment of cells to prevent or slow the change of the cells from a normal phenotype to a transformed malignant phenotype. Ceramide is able to induce differentiation of malignant cells and otherwise undifferentiated cells, thus the compounds of the invention are believed to be useful in inducing and slowing the proliferation of early (clinically undetectable) malignant cells. This would constitute a strong chemopreventive agent. Similarly, retinoic acid may be useful as a chemopreventive agent, thus since retinoic acid elevate levels of ceramide, the compounds of the invention may also be useful for chemoprevention along this route.

The compounds of the present invention and/or their pharmaceutically active salts may be formulated into

pharmaceutical compositions or medicaments which may be used to treat mammals such as man, which are afflicted with the various conditions described herein and others which are caused by defective differentiation processes. The pharmaceutical compositions or medicaments preferably contain therapeutically effective amounts of the compounds of the present invention and/or their pharmaceutically acceptable salts. The compounds of the invention may be administered to a mammal having the disease, or suspected of having the disease, singly or in combination with other compounds of the invention or other therapeutic or palliative agents. The compositions of the present invention may be administered in any mode, such as orally, parenterally, intradermally, intramuscularly, intravenously, subcutaneously or topically. The actual mode can readily be determined by analogy to known methodologies and will depend on the particular disease state being treated, its severity, and the age and condition of the patient. They may be administered orally in tablet, capsule, or elixir form, or parenterally in the form of a solution or suspension. For injection purposes, the medium used is preferably a sterile liquid. As an injection medium, it is preferred to use water which contains the stabilizing agents, solubilizing agents and/or buffers conventional in the case of injection solutions. Desirable additives include, for example, tartrate and borate buffers, ethanol, dimethylsulfoxide, complex forming agents (for example, ethylenediaminetetraacetic acid) high molecular weight polymers (for example polyethylene oxide) for viscosity regulation or polyethylene derivatives of sorbitan anhydrides.

The total routine (e.g., daily, weekly, monthly, etc.) dose of the compounds according to the present invention will be that effective to result in differentiation of the affected cells, a reduction in cell proliferation, or an improvement or stabilization of the condition being treated. One of skill in the art can readily ascertain the optimum therapeutically effective dosage to use for a particular case, using as a starting point the range delineated above.

When a composition for the treatment of a disease is prepared or manufactured, a compound or a physiologically acceptable salt of a compound according to this invention or a mixture thereof may be shaped together with a physiologically acceptable vehicle, carrier, excipient, binder, preservative, stabilizer, flavoring, and/or additive, into a unit dosage form. Typical examples of additives that can be used in tablets and capsules are binders such as tragacanth gum, gum arabic, corn starch and gelatin; excipients such microcrystalline cellulose, sealing agents such as corn starch, pre-gelatinized starch and alginic acid; lubricants such as magnesium stearate; sweeteners such as sucrose, lactose, and aspartase; and flavorings such as peppermint. Other additives include edible oil as a liquid carrier such as in capsules, shellac, sugar and combinations thereof such as in tablet coating).

Parenteral injection may employ, as a vehicle to dissolve or suspend the active ingredient, water, natural vegetable oils such as sesame oil, coconut oil, peanut oil and cottonseed oil, and synthetic oils such as ethyl oleate, and may contain buffering agents, preservatives and anti-oxidants as required.

The method of this invention may be carried out by directly contacting in effective amount of a composition according to the invention with cells. However, it is also possible, and within the scope of the invention, to carry out the method indirectly, e.g., by administering a compound or composition which has an *in vivo* activity of inducing production of one of the compounds of this invention. Further, pro-drug precursors which are converted *in vivo* to a compound of the invention are also within the scope of the invention.

EXPERIMENTAL

Some of the compounds referred to in the present Specification are named by a convenient shorthand to reflect the total number of carbon atoms in the carbon chains of the compounds of formula I. For example, C_{18}/C_2 ceramide refers

to the compound of formula I wherein R_1 is C_{15} alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5 and R_5 is methyl. The double bond in R_1 in C_{18}/C_2 ceramide is in the same position as the double bond in ceramide. Thus there are 18 carbons in the first mentioned chain which chain contains the carbons in R_1, the carbon to which R_2 is attached, the carbon to which N is attached and the CH_2OH group at the end of the chain, and there are 2 carbons in the second-mentioned chain, i.e., the amide carbon of R_4 and the methyl carbon of R_5. The compounds are referred to in this manner, with the first-mentioned carbon chain comprising R_1 and the carbons to which R_2, N, and OH are attached, and the second-mentioned carbon chain referring to the carbons in R_4. Similarly C_{18}/C_6 ceramide refers to the compound of formula I wherein R_1 is C_{15} alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5 and R_5 is pentanyl. C_{11}/C_8 ceramide refers to the compound wherein R_1 is C_8 alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5 and R_5 is heptanyl. In each of these examples, the double bond in R_1 is in the same position as in ceramide although other sites of unsaturation may be used. 3-O-methyl sphingosine refers to the compound of formula I wherein R_1 is C_{15} alkenyl, R_2 is methoxy, R_3 is H, R_4 is COR_5 and R_5 is methyl.

1δ,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) was obtained from Hoffman-LaRoche, Nutley, New Jersey. SM and PC were purchased from Avanti Polar Lipids while ceramide was purchased from Supelco. Insulin, transferrin, NBT and α-naphthyl acetate were purchased from Sigma Chemical Co., St. Louis, Missouri.

PREPARATION OF CERAMIDE AND SPHINGOSINE ANALOGS

N-acetyl and [^{3}H] N-acetylsphingosine were synthesized as described in Gaver, R.C. and Sweely, C.C., *J. Amer. Chem. Soc.* 88:3643-3647 (1966). Briefly, N-acetylsphingosine (C_{18}/C_2 ceramide) and N-hexanoylsphingosine (C_{18}/C_6 ceramide) were prepared by acylation of neutral sphingosine by acetic anhydride and caproic anhydride, respectively (yield =90%). N-acetyl [^{3}H] sphingosine (specific activity 2.5 X 10^4 cpm/nmol) was prepared by

reduction of 3-oxo-1-hydroxy-2-acetamide-4-octadecene, which was obtained by oxidation of cold N-acetylsphingosine with dry chromium anhydride in pyridine/benzene (yield 80% after TLC preparation), with [^{3}H]$NaBH_4$ (yield =40% after TLC preparation). N-ethylsphingosine was prepared by reduction of N-acetylspingosine with $LiBH_4$ and purified by preparative TLC (yield 30%). C_{11}/C_8 ceramide was prepared according to the method of Liotta et al. Tetrahedrom Letters 29: 3037 (1988). All structures were verified by NMR, and purity was established by TLC and estimated to exceed 97%. These compounds were dissolved with ethanol and delivered in media (final concentration of ethanol was less than 0.1%).

PREPARATION OF 3-O-METHYLSPHINGOSINE

3-O-methylsphingosine was synthesized as described in Carter, et al., *Journal of Biochemistry* 192: 197-207 (1951). 100 mg of beef brain cerebrosides (Catalog No. A-46, Serdary Research Labs, London, Ontario, Canada) was dissolved in 112μl concentrated sulfuric acid/2.3ml methanol in a round bottom flask. The mixture was heated and refluxed while stirring for 6 hours. Following heating, the reaction mixture was cooled on ice for approximately 15 minutes. Precipitates of fatty acids and methyl esters were removed by filtering thorugh #1 Whatman paper on a Büchner funnel. Filtrate was extracted four times with 1 ml petroleum ether (each extraction) to remove remaining fatty acids and esters. Ether was removed under vacuum for approximately 15 minutes. The solution was neutralized with 4N Methanolic KOH (~6.5 ml judging by pH paper) and precipitated potassium sulfate was filtered off on #1 Whatman paper on a Büchner funnel. The filtered solution was stored at 4°C (refrigeration) overnight and filtered to remove additional potassium sulfate precipitate which formed overnight. 6N NaOH was added to the solution to pH 10 judging by pH paper. The solution was then extracted with diethyl ether two times. The ether extracts were combined, washed once with water and dried over sodium sulfate, under vacuum. Dried extracts were dissolved in chloroform:methanol (1:1).

The sample was purified by thin layer chromatography (TLC) as described in Sambasivaroco and McCluer, *Journal of Lipid Research* 4:106-108 (1963). A preparative TLC plate was used to separate the product using chloroform:methanol:2N ammonium hydroxide (40:10:1) as the solvent, and sphingosine as a marker. A small portion of the plate was visualized using ninhydrin spray. 3-O-methyl-spingosine runs high on the plate and was found to be the fastest migrating component. The silica band corresponding to 3-O-methylsphingosine was scraped from the plate and 3-O-methylsphingosine was eluted from the silica with chloroform:methanol (1:1). The elutant was spun in a centrifuge (IEC) at 2,000 rpm for approximately 5 minutes and the supernatant was drawn off into a clean tube. Elution of 3-O-methylsphingosine from the silica was repeated with chloroform:methanol (1:1) and the chloroform:methanol supernatants were combined. The combined supernatants were dried under vacuum and resuspended in ethanol for a total yield of 11mg of 3-O-methylsphingosine.

CELL CULTURE

Human myelocytic leukemia HL-60 cells (45 passages) were obtained and grown in RPMI 1640 medium (Sigma Chemical Co., St. Louis, Missouri) containing 10% fetal calf serum at 37°C in 5% CO_2 incubator. The cells were washed twice with phosphate buffered saline (PBS) and resuspended in serum-free media containing insulin (5mg/liter) and transferrin (5 mg/liter) before treatment with various compounds.

MASS MEASUREMENTS OF LIPIDS

After harvesting the cells at the indicated times, the lipids were extracted by the method of Bligh and Dyer, *Can. J. Biochem. Physiol.* 37:911-917 (1959). The samples were dried down under nitrogen gas and dissolved with 0.1 ml chloroform: 40 µl was applied to a thin layer chromatography (TLC) plate (Merck); and 40 µl was used for measurement of phospholipid phosphate (duplicate measurement). Phosphate was measured as described in Van Veldhoven, P. and Mammaerts, G., *Ann. Biochem.* 161:45-48 (1987). To identify sphingomyelin (SM) and phosphorylcholine (PC), TLC plates were developed in

chloroform/methanol/acetic acid/H_2O (50/30/8/5) (solvent A) or chloroform/methanol/2N NH_4OH (60/35/5) (solvent B). The combination of solvents A and B were used for two-dimensional TLC. After staining the plates with iodine vapor, the spots corresponding to SM and PC were scraped, extracted with chloroform/methanol (1:1), dried down under nitrogen, and phospholipid phosphate was measured.

CERAMIDE MEASUREMENTS

The mass of ceramide was measured enzymatically using sn-1,2-diacylglycerol (DAG) kinase as described in Preiss et al., *J. Biol. Chem.* 261:8697-8700 (1986) and Van Veldhoven, *Anal. Biochem.* 183: 177-189 (1989). To confirm the conversion of ceramide in HL-60 cells to ceramide 1-phosphate, ceramide phosphate and phosphatidic acid were separated on TLC by different solvent systems such as chloroform/methanol /acetic acid (65/15/5), chloroform/pyridine/formic acid (60/30/8), and chloroform/acetone/methanol/acetic acid/H_2O (6/2/2). Rf values were compared with those of reference standard ceramide. Additionally, ceramide 1-phosphate was converted to sphingosine 1-phosphate by alkaline hydrolysis (70°C, twenty hours in 1N NaOH) and the Rf value was compared with that of reference sphingosine 1-phosphate in the solvent containing butanol/acetic acid/H_2O (6/2/2) or chloroform/ acetone/methanol/acetic acid/H_2O (10/4/2/2/1) and found to be identical at 0.45 or 0.42.

ANALYSIS OF HL-60 CELL GROWTH AND DIFFERENTIATION

Cell growth was quantified using a hemocytometer. Cell viability was judged by the ability to exclude tryan blue. Viability was always more than 80% unless otherwise described. Nitro blue tetrazolium (NBT) reducing ability was used as a marker for both macrophage/monocyte and granulocyte lineage and non-specific esterase was used as a marker for macrophage/monocyte lineage. Both markers were measured as described in Okazaki, T. et al., *J. Cell Physiol.* 131:50-57 (1987).

UPTAKE AND METABOLISM OF [^{3}H]$C_{18}C_2$ CERAMIDE:

After resuspending in serum-free media, HL-60 cells

were labeled with [^{3}H]C_{18}/C_2 ceramide (1 x 10^5 cpm/ml), harvested at the indicated times, and washed three times with PBS. Lipids were then extracted by the method of Bligh and Dyer, *supra*. The dried-down samples were dissolved with 100 µl chloroform; 20 µl were applied on TLC plates and 40 µl were used for measuring phospholipid phosphate. The plates were developed in the solvent containing chloroform/methanol/2N NH_4OH (40/10/1) to separate C_{18}/C_2 ceramide, SM and sphingosine (SPH). The spots were scraped and counted in Safety Solve (Research Products International Corp.) in a LKB scintillation counter (LKB).

DOSE AND TIME DEPENDENCE OF CERAMIDE FORMATION IN RESPONSE TO 25-$(OH)_2D_3$:

The mass of ceramide was measured by adapting the diacylglycerol-kinase assay previously developed to quantitate diacylglycerol (see Van Veldhove et al *supra*). The E. coli DAG kinase is able to quantitatively convert ceramide to ceramide 1-phosphate. Cellular ceramide was identified following conversion to ceramide 1-phosphate by comigration with standard ceramide phosphate on thin layer chromatography (TLC). Cellular and reference ceramide phosphate showed identical Rf values when TLC plates were developed in 4 different solvent systems. Further identification was achieved by alkaline hydrolysis of ceramide phosphate. This resulted in the formation of sphingosine 1-phosphate which comigrated (Rf = 0.45) with standard on TLC developed in butanol/acetic acid/H_2O (6/2/2) solvent system.

As shown in Figure 1, treatment of HL-60 cells with 1,25-$(OH)_2D_3$ resulted in a dose-dependent elevation in ceramide levels at 2 hours following treatment. The data are shown as % of control (in the absence of C_{18}/C_2 ceramide). Bars represent one standard derivation for duplicate measurements. Baseline ceramide levels were 26.1 ± 0.82 nmol/nmol phospholipid. The results are representative of three different experiments.

Maximal ceramide elevations occurred at 100 nM 1,25-$(OH)_2D_3$. At this concentration of 1,25-$(OH)_2D_3$, there was a

41% increase in ceramide mass over control levels. The dose-dependence of ceramide formation on 1,25-$(OH)_2D_3$ closely paralleled the dose-dependence of HL-60 cell differentiation on 1,25-$(OH)_2D_3$ which peaks at 100-300 nM. These results, therefore, suggest a quantitative relationship between ceramide formation and cell differentiation.

The time dependence of ceramide levels in response to the action of 1,25-$(OH)_2D_3$ on HL-60 cells was also investigated. As shown in Figure 2, HL-60 cells were harvested at the indicated time points after treatment with 100 nM C_{18}/C_2 ceramide. Ceramide mass was measured as described in the Experimental Procedures. The results were obtained from two determinations. Bars represent one standard deviation. Data are representative of three different experiments when HL-60 cells were treated with 100 nM 1,25-$(OH)_2D_3$ (an optimal concentration from the dose-response illustrated in Fig. 1), ceramide levels progressively increased over the first 2 hours and then returned to baseline. The earliest increase was detected at 30 min following 1,25-$(OH)_2D_3$ treatment (7% over baseline) and peaked at 2 hours with a 41% increase (Fig. 2).

These studies show that 1,25-$(OH)_2D_3$ incudes a time- and dose-dependent transient increase in ceramide levels which clearly precedes the onset of differentiation of HL-60 cells (peak ceramide formation occurs at 2 hours with phenotypic changes of differentiation occurring at 2-4 days.). Since ceramide formation appears to be one of the earliest biochemical changes in response to 1,25-$(OH)_2D_3$, these studies suggest a role for ceramide as a lipid mediator.

DERIVATION OF CERAMIDE FROM SPHINGOMYELIN:

In previous studies, Okazaki, T. et al., supra, it was shown that 1,25-$(OH)_2D_3$, induced hydrolysis of sphingomyelin with simultaneous changes in phosphorylcholine and ceramide levels; followed by resynthesis of sphingomyelin to baseline levels. To confirm that ceramide was quantitatively generated from sphingomyelin, the mass of hydrolyzed sphingomyelin was measured and compared to the

mass of generated ceramide. As seen from Figure 2, the mass of ceramide peaked at 2 hours with a net formation of 13±2 pmol ceramide per nmol phospholipid. Ceramide levels then returned to baseline at four hours. As shown in Figure 3, the total levels of phospholipids (Fig. 3A) and phophatidylcholine (Fig. 3B) did not significantly change over the first 4 hours following $1,25-(OH)_2D_3$ treatment. However, sphingomyelin levels decreased from 51±6 pmol/nmol phospholipid to 34±2 pmol/nmol phospholipid (Fig. 3C) at 2 hours. The net decrease in sphingomyelin mass of 17±4 pmol/nmol phospholipid is very close to the net increase in ceramide mass levels of 13±pmol/nmol phospholipid. These results strongly suggest that ceramide is generated from sphingomyelin breakdown in response to $1,25-(OH)_2D_3$ action on HL-60 cells. Labeling of sphingolipid precursors with [^{3}H]palmitate showed that no other sphingolipid underwent significant changes in response to $1,25-(OH)_2D_3$ during this time interval and the sphingomyelin pool constituted the largest labeled pool among the various sphingolipids. Therefore, it is unlikely that ceramide could be derived from the hydrolysis of sphingolipids other than sphingomyelin. These studies, however, do not rule out the possibility of de novo synthesis of ceramide in response to $1,25-(OH)_2D_3$ although this is unlikely in the face of significant and commensurate hydrolysis of sphingomyelin.

These data also show that total phospholipids in HL-60 cells were 15.6±1.0 fmol/cell with PC and SM accounting for 53.3±2.3% and 5.1±0.6% of total phospholipids, respectively, These are very close to previous data showing a SM/PC ration of 0.05 - 0.10 using [^{3}H] choline to label the two lipids. Okazaki, T. et al., *J. Biol. Chem.* 264:19076-19080 (1989). These results also explain why no significant change in total phospholipid was detected since the net decrease in SM levels corresponds to only 1.5 - 2.0% change in total phospholipid levels.

C_{18}/C_2-CERAMIDE POTENTIATES THE EFFECTS OF $1,25-(OH)_2D_3$ ON HL-60 CELL DIFFERENTIATION:

It has been shown that the exogenous application of

bacterial sphingomyelinase, which induces hydrolysis of membrane sphingomyelin and the formation of ceramide, potentiates the ability of 1,25-$(OH)_2D_3$ to induce HL-60 cell differentiation. Okazaki, T. et al., *supra*. The use of bacterial sphingomyelinase, however, was complicated by the hydrolysis of membrane phosphatidyl-choline (PC) at higher concentrations of sphingomyelinase, thus limiting interpretations.

To overcome this problem and to directly test whether ceramide can mimic the action of bacterial spingomyelinase, a synthetic cell permeable ceramide, C_{18}/C_2 ceramide, having an acetate in amide linkage was prepared. Compared to naturally occurring ceramides, C_{18}/C_2 ceramide has 14-16 less carbons, and therefore, displays higher water solubility. Under similar conditions, naturally occurring ceramide with long N-acyl chains, at 50 μM, did not affect cell growth or cell differentiation consistent with the poor uptake of long-chain N-acyl ceramides.

This is analogous to cell-permeable DAG analogs such as dioctanoylglycerol and oleoylacetylglycerol which have shorter acyl chains than naturally occurring DAGs. When HL-60 cells were treated simultaneously with suboptimal concentrations of 1,25-$(OH)_2D_3$, (1 nM which is 100-fold lower than the optimal concentration and various concentrations of C_{18}/C_2 ceramide), enhancement of cell differentiation was observed.

HL-60 cells (2.5×10^5 cells/ml) were treated simultaneously with various concentrations of C_{18}/C_2 ceramide and 1 nM 1,25-$(OH)_2D_3$. Cell differentiation was judged by NBT reducing ability (shaded bars) and NSE activity (unshaded bars). The effects of C_{18}/C_2 ceramide on cell growth are shown in the inset. The results were obtained from three different experiments. Bars represent one standard deviation.

As shown in Figure 4, C_{18}/C_2 ceramide showed a dose-dependent enhancement of 1,25-$(OH)_2D_3$ differentiation with peak effect occurring at 1 μM. On day 4 following treatment, 1 μM C_{18}/C_2 ceramide caused an increase of NBT reducing

activity and non-specific esterase (NSE) activity from 11.9±2.9% to 57.8±1.4% and from 2.2±0.9 to 39.2±7.2%, respectively. Over the same concentration range, C_{18}/C_2 ceramide induced mild inhibition of cell growth (Fig 4, inset) without significant effects on cell viability. Cell viability was always greater than 80%. Although there was mild inhibition of cell growth, the absolute numbers of NBT and NSE positive cells induced by 1 μM of C_{18}/C_2 ceramide increased from 0.9 x 10^5 cell/ml to 2.54 x 10^5 cells/ml and from 0.16 x 10^5 cells/ml to 1.72 x 10^5 cells/ml on day 4, compared to control.

CELL PERMEABLE CERAMIDES INDUCE HL-60 CELL DIFFERENTIATION INDEPENDENT OF 1,25-$(OH)_2D_3$.

The strong synergy between subthreshold concentrations of 1,25-$(OH)_2D_3$ and low concentrations of C_{18}/C_2 ceramide (100 nM-1 μM) suggested that higher concentrations of C_{18}/C_2 ceramide may induce differentiation independent of 1,25-$(OH)_2D_3$ addition. The effects of synthetic C_{18}/C_2 ceramide on cell growth and differentiation were therefore examined. Treatment of HL-60 cells with increasing concentrations of C_{18}/C_2 ceramide resulted in a dose-dependent inhibition of cell growth as shown in Figure 5. The various concentrations of C_{18}/C_2 ceramide are represented as follows: 0 - Control; closed diamond - 1 μM; closed square - 3 μM; closed circle -6 μM and closed triangle - 10 μM. The results were obtained from three determinations. Bars represent one standard deviation. Ten μM of C_{18}/C_2 ceramide caused severe loss of cells by day 7. The loss of cells may be, at least in part, due to the induction of cell differentiation by C_{18}/C_2 ceramide rather than to simple toxicity since more than 30% of cells were induced to differentiate by day 2 of treatment.

As shown in Figure 6, increasing concentrations of C_{18}/C_2 ceramide caused a progressive increase in differentiation of HL-60 cells by 4 days as quantitated by NBT-reducing ability and induction of NSE activity. NBT activity is represented by shaded bars, and NSE activity is represented

by open bars. The results are averages of three determinations. Bars represent one standard deviation. The cells were treated with various concentrations of C_{18}/C_2 ceramide for 4 days. NBT positive cells increased from 0±1.0 to 53.2±1.6%, and NSE-positive cells increased from 1.0±1.9% to 46.4±6.0% following treatment with 6 μM of C_{18}/C_2 ceramide. Significant increases in differentiated cells were also observed with concentration of C_{18}/C_2 ceramide as low as 1 μM.

Examination of the morphologic phenotype of HL-60 cells treated with ceramide showed morphological changes consistent with the monocytic phenotype induced by 1,25-$(OH)_2D_3$. These cells were characterized by a larger cytoplasmic to nuclear ratio, lobulated and eccentric nucleus, and disappearance of nuclear bodies and azurophilic granules. The cells also acquired NBT-reducing ability, and NSE activity, the latter being a specific marker of monocytic differentiation.

The time-dependence of differentiation in response to C_{18}/C_2 ceramide was next examined using 6 μM C_{18}/C_2 ceramide as an optimal concentration that produces maximal differentiation with minimal cytotoxicity. Cell differentiation was judged by NBT reducing ability and NSE activity. The addition of 6μM C_{18}/C_2 ceramide caused a progressive increase in NBT and NSE positive HL-60 cells with up to 61% and 56% respectively, by day 7 of treatment, as shown in Figure 7. In Figure 7, HL-60 cells treated with 6 μm ceramide are represented by closed figures and cells not treated with ceramide are represented by open figures. NBT reducing ability is represented by and circles. NSE activity is represented by squares.

These results show that the addition of C_{18}/C_2 ceramide alone can induce HL-60 cell differentiation into a monocytic phenotype with 6μM C_{18}/C_2 ceramide displaying similar effectiveness as 1,25-$(OH)_2D_3$. At an optimal concentration of 100 nM, C_{18}/C_2 ceramide induces NBT reducing ability in 74±6% of cells and NSE in 51±4% of cells compared to 53.2±1.6% and 46.4±6% for C_{18}/C_2 ceramide on day 4.

As shown in Figure 8, the addition of C_{18}/C_2 ceramide to HL-60 cells did not modulate cellular levels of sphingomyelin. HL-60 cells were treated with 5 μM C_{18}/C_2 ceramide for the indicated times. Sphingomyelin was extracted and measured as described in "Experimental Procedure". The data are shown as % of control (in the absence of C_{18}/C_2 ceramide). Bars show one standard deviation. The results were obtained from two different experiments. These results strongly suggest that the effects of C_{18}/C_2 ceramide and bacterial sphingomyelinase (SMase) on HL-60 cell differentiation are mediated by ceramide and not by the changes in SM levels *per se*.

To further evaluate the role of ceramide as a second messenger, experiments were performed to examine whether a short duration of exposure of HL-60 cells to ceramide is sufficient for induction of differentiation. C_{18}/C_2 ceramide, added to cells, could be back-extracted into media following repeated washing of cells, so that after 3 washes, less than 20% of original C_{18}/C_2 ceramide remained in association with the cell pellet.

Since 1,25-$(OH)_2D_3$ caused elevation of endogenous ceramide over approximately 2 hrs, HL-60 cells were exposed to C_{18}/C_2 ceramide (0.5 - 2μM) for 2 hr. C_{18}/C_2 ceramide was back-extracted, and differentiation was evaluated. The cells were treated without or with C_{18}/C_2 ceramide (0.5, 1 or 2 μM) for two (Figures 9 and 10) or four hours (Figure 10), washed with RPMI 1640 media three times and then resuspended in serum-free RPMI 1640 media. At the indicated day (Figure 9) or four days (Figure 10) after washing out treatment, the differentiation was measured by NBT reducing ability as described in "Experimental Procedure". Bars mean one standard deviation. In Figure 9, open circles represent 0 μM C_{18}/C_2 ceramide. Closed triangle represent 0.5 μM C_{18}/C_2 ceramide. Closed squares represent 1 μM C_{18}/C_2 ceramide. Closed circles represent 2 μM C_{18}/C_2 ceramide. In Figure 10, 0 μM C_{18}/C_2 ceramide is represented by unshaded bars. 0.5 μM C_{18}/C_2 ceramide is represented by light cross-hatched bars. 1 μM

C_{18}/C_2 ceramide is represented by dark cross hatched bars. 2 μM C_{18}/C_2 ceramide is represented by shaded bars. The results were obtained from two different experiments. The results were obtained from two different experiments. C_{18}/C_2 (1 or 2 μM) caused significant differentiation of HL-60 cells under these conditions (Figure 9) indicating that a 2-hr exposure of HL-60 cells to C_{18}/C_2 ceramide was sufficient for induction of differentiation. A 4-hr exposure did not result in significantly more differentiation (Figure 10). These studies suggest that a short exposure of HL-60 cells to elevated ceramide levels is sufficient for commitment to differentiation.

EFFECTS OF CERAMIDE AND SPINGOSINE ANALOGS ON D_3-INDUCED HL-60 CELL DIFFERENTIATION.

Sphingosine is a pharmacologic inhibitor of protein kinase C activity *in vitro* and in different cell systems. Because ceramide can potentially be metabolized to sphingosine by the action of acid and/or neutral ceramidases, we investigated whether the actions of ceramide on HL-60 cells could be attributed to the formation of sphingosine. No sphingosine could be detected following treatment of HL-60 cells with $1,25\text{-}(OH)_2D_3$. Moreover, the addition of C_{18}/C_2 ceramide to HL-60 cells did not result in any measurable sphingosine formation. For these experiments, C_{18}/C_2 was labeled with $[^3H]$ on the third carbon of the sphingosine base. Cells (5×10^6 cells/ml) were labeled with 4 μM $[^3H]C_{18}/C_2$ ceramide (1×10^5 cpm/ml). C_{18}/C_2 ceramide was delivered in ethanol. The additional $[^3H]C_{18}/C_2$ ceramide to HL-60 cells resulted in prompt uptake of labeled C_{18}/C_2 ceramide but no conversion to sphingosine as shown in Figures 11 and 12. The uptake of C_{18}/C_2 ceramide was about 20% at 0.5 hr after treatment. The rest of the ceramide (80%)remained unchanged in the medium. Figure 11 shows an autoradiography of a TLC plate (24 h exposure). In Figure 12 lipids were extracted, separated by TLC plates and radioactivity was counted as described in "Experimental Procedures". Ceramide is represented by open circles. Sphingomyeline is represented by open squares.

Sphingosine is represented by closed circles.

Also, the addition of 1,25-$(OH)_2D_3$ to C_{18}/C_2 ceramide-labeled cells did not result in the formation of sphingosine. A small percentage of label was converted to sphingomyelin (2.8% at 12h after labeling). These studies show that 1,25-$(OH)_2D_3$ does not lead to the formation of sphingosine and that exogenous ceramide analogs, when added to cells (in the presence or absence of 1,25-$(OH)_2D_3$), are not metabolized to sphingosine.

Sphingosine is slowly metabolized in HL-60 cells by primarily becoming incorporated into ceramide and other sphingolipids. Merrill, A.H., et al., *J. Biol. Chem.* 261:12610-12615 (1986). However, the above studies do not rule out rapid metabolism of sphingosine generated from ceramide, thus escaping detection by the above methods. Since the objective was to evaluate the role of sphingosine in mediating the effects of ceramide, the ability of sphingosine to mimic the action of ceramide was therefore tested.

As shown in Figure 13, when HL-60 cells were treated simultaneously with various concentrations of sphingosine and suboptimal concentrations of 1,25-$(OH)_2D_3$ (1nM) for 4 days, NBT reducing ability and NSE activity did not change compared to control. HL-60 cells (2.5×10^5 cells/ml) were treated with various concentrations of sphingosine for 4 days in the presence of 1nM 1,25-$(OH)_2D_3$. Cell differentiation was judged by NBT reducing ability (shaded bar) and NSE activity (open bar). The effects of sphingosine on HL-60 cell growth are shown in the inset. The results were obtained from three determinations. Bars represent one standard deviation.

These studies show that sphingosine does not enhance the ability of 1,25-$(OH)_2D_3$ to induce HL-60 cell differentiation in clear distinction from the effects of ceramide (compare Figs. 4 and 13). [^{3}H]sphingosine was taken up efficiently by HL-60 cells demonstrating that the lack of effects of sphingosine were not due to poor uptake. Moreover, sphingosine did slow the growth of HL-60 cells (Fig. 13 inset) to a level comparable to that induced by C_{18}/C_2 ceramide

indicating a cellular effect of sphingosine other than the induction of differentiation. The ability of sphingosine to slow HL-60 growth without enhancing differentiation also supports the notion that ceramide is primarily acting to induce differentiation independent of growth rate of HL-60 cells. To further support a role for ceramide in cell differentiation, independent of sphingosine, synthetic ceramide and sphingosine analogs were prepared and tested for their ability to enhance HL-60 cell differentiation by suboptimal concentrations of $1,25\text{-}(OH)_2D_3$. As shown in Table 1, both C_{18}/C_6 ceramide and C_{18}/C_2 ceramide caused significant enhancement of NBT reducing ability and NSE activity comparable to that observed with 100 nM $1,25\text{-}(OH)_2D_3$. C_{11}/C_8 also caused significant enhancement of NBT reducing ability and NSE activity. Studies with C_{11}/C_8 ceramide are particularly relevant in ruling out an important role for sphingosine. Deacylation of C_{11}/C_8 ceramide would result in the formation of a C_{11}-sphingosine analog which has been shown to lace the *in vitro* and cellular effects of sphingosine. Norjiri, H., et al., *supra*.

TABLE 1 Effects of various sphingolipids on HL-60 cell differentiation treated with 1 nM 1,25-$(OH)_2D_3$

Treatment	Concentration of sphingolipid (μM)	Cell Number ($x10^5$cells/ml)	NBT positive cells %	NSE positive cells %
1 nM 1,25-$(OH)_2D_3$	0	7.6 ± 1.2	11.9 ± 2.9	2.2 ± 0.9
C18/C2 ceramide	0.1	7.6 ± 1.0	35.4 ± 4.8*	26.5 ± 2.4*
+ 1 nM 1,25-$(OH)_2D_3$	1.0	4.4 ± 0.1	57.8 ± 1.4*	39.2 ± 7.9*
C18/C6 ceramide	0.1	7.2 ± 1.8	33.5 ± 0.5*	18.3 ± 4.5*
+ 1 nM 1,25-$(OH)_2D_3$	1.0	6.8 ± 1.6	58.1 ± 1.5*	29.6 ± 1.8*
N-ethylsphingosine	0.1	5.0 ± 1.4	16.5 ± 0.5	1.3 ± 0.3
+ 1 nM 1,25-$(OH)_2D_3$	1.0	4.2 ± 1.2	17.8 ± 4.5	1.0 ± 1.0
sphingosine	0.1	7.8 ± 1.6	11.3 ± 0.5	2.5 ± 1.0
+ 1 nM 1,25-$(OH)_2D_3$	1.0	5.6 ± 0.6	10.7 ± 1.7	2.5 ± 1.0
C_{11}/C_8 ceramide	0.1	-	25*	18*
+ 1nM 1,25-$(OH)_2D_3$	1.0	-	38.5*	34.5*

HL-60 cells (2.5 x 10^5 cells/ml) were treated simultaneously with the indicated lipid and subthreshold 1,25-$(OH)_2D_3$ (1nM) for four days. The results were obtained from three determinations. Asterisks show that the difference from the control (1nM 1,25-$(OH)_2D_3$) is significant at a p value <0.01.

On the other hand, sphingosine failed to induce any significant changes in those two parameters of cell differentiation. Similar results with sphingosine have also been noted. Stevens, V.L., et al. *Cancer Res.* 49:3229-3234 (1989). Moreover, N-ethyl sphingosine, which is a potent inhibitor of protein kinase C, failed to increase NBT and NSE activity significantly over baseline. These studies show that ceramide derivatives, but not sphingosine and its analog, enhance 1,25-$(OH)_2D_3$ HL-60 cell differentiation.

In a previous study, it was discovered that 1,25-$(OH)_2D_3$ caused hydrolysis of sphingomyelin in HL-60 cells with the concomitant generation of ceramide and phosphorylcholine in what appeared to be a regulated "sphingomyelin cycle". Okazaki, T., et al., *J. Biol. Chem.* 254:19076-19080 (1989). Sphingomyelin hydrolysis was suggested to play a role in HL-60 cell differentiation since the addition of exogenous bacterial sphingomyelinase potentiated the ability of subthreshold concentrations of 1,25-$(OH)_2D_3$ to induce cell differentiation.

Applicants have now discovered that ceramide functions as a lipid mediator transducing the effects of 1,25-$(OH)_2D_3$ on HL-60 cell differentiation. Low concentrations of ceramide (100 nM - 3 μM) enhanced the ability of subthreshold concentrations of 1,25-$(OH)_2D_3$ to induce cell differentiation. More importantly, higher concentrations of ceramide (1-6 μM) were able to induce HL-60 cell differentiation in the absence of 1,25-$(OH)_2D_3$. The phenotype of differentiated HL-60 cells closely resembles the monocytic phenotype induced by 1,25-$(OH)_2D_3$. These studies strongly suggest that ceramide may play an essential role in mediating the action of 1,25-$(OH)_2D_3$ on cell differentiation. Moreover, C_{18}/C_2 ceramide was effective in causing differentiation when cells were exposed to it for only 2 hrs. This strongly suggests that the ceramide response to 1,25-$(OH)_2D_3$ action is sufficient for the induction of differentiation. Also, since approximately 20% of added ceramide was taken up by cells, the results indicate that the effective concentration of C_{18}/C_2 ceramide is the nM

range (20-1000 nM).

At the present time, Applicants do not know the mechanism by which ceramide mediates the effects of 1,25-$(OH)_2D_3$ on cell differentiation. No immediate target for ceramide action could be identified. Because ceramide may serve as a precursor to sphingolipids and sphingosine, its action may be mediated by metabolites. The ganglioside GM_3 has been reported to increase in response to phorbol ester-induced HL-60 cell differentiation and also to induce cell differentiation along a monocytic lineage. Norjiri, H. et al., *Proc. Natl. Acad. Sci.* 83:782-786 (1986). Ceramide may serve as a precursor to gangliosides such as GM_3. However, 1,25-$(OH)_2D_3$ was found not to modulate GM_3 (Nojiri, H. et al. (1988) Proc. Natl. Acad. Sci. USA 83: 782-786), and Applicants found very little ceramide converted to gangliosides. Moreover, the dose response of HL-60 cells to ceramide is much lower than that reported for GM_3 (raising the possibility that the action of GM_3 may be due to its further metabolism to ceramide).

Ceramide may also serve as a precursor to sphingosine which would be generated through a single hydrolysis step by the action of neutral or acid ceramidases. Applicants' data strongly argue against a role for sphingosine for mediating the effects of ceramide. This is supported by: 1) no sphingosine could be detected in response to the action of 1,25-$(OH)_2D_3$ on HL-60 cells; 2) sphingosine did not enhance the ability of 1,25-$(OH)_2D_3$ to induce HL-60 cell differentiation nor did it cause monocytic differentiation of HL-60 cells on its own; 3) other ceramide derivatives were able to induce HL-60 cell differentiation but sphingosine and its related analog N-ethyl sphingosine failed to enhance 1,25-$(OH)_2D_3$ induced differentiation, and 4) C_{11}/C_8 ceramide, whose hydrolysis results in a short chain sphingosine that does not inhibit protein kinase C (Merril, A.H. et al. (1989) Biochemistry 28: 3138-3145) was as effective as C_{18}/C_2 ceramide in inducing cell differentiation.

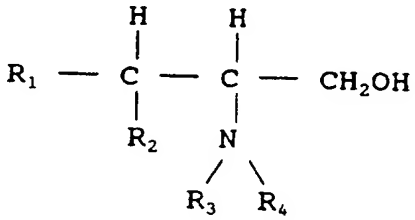
Studies with $C_{11}C_8$ ceramide are particularly relevant in ruling out an important role for sphingosine. Deacylation

of $C_{11}C_8$ ceramide would result in the formation of a C_{11}-sphingosine analog which has been shown to lack the *in vitro* and cellular effects of sphingosine. Norjiri, H., et al., *supra*.

Since these two pathways appear unlikely, ceramide may have other targets mediating its actions. Sphingomyelin serving as cellular reservoir acted upon by sphingomyelinase to produce ceramide, a potential lipid mediator (second messenger) is analogous to the glycerolipids serving as cellular reservoirs acted upon by phospholipase C to produce diacylglycerol second messengers.

WHAT IS CLAIMED IS:

1. A method of inducing cell differentiation, comprising contacting a cell capable of undergoing differentiation with a compound having the formula



wherein R_1 is C_1 to about C_{20} alkyl or alkenyl;

R_2 is hydroxyl, alkoxy or H,

R_3 is H or lower alkyl;

R_4 is COR_5, SO_2R_5, or CSR_5, where R_5 is C_1 to C_{20} alkyl, alkenyl, or alkynyl, which may be substituted by one or more of the following functional groups: OH, SH, OR_6, SR_6, NR_7R_8, $COOR_9$, and $CONR_{10}R_8$, where R_6, R_7, R_8, R_9, and R_{10} independently are H, alkyl, aryl, alkaryl and arylalkyl up to about 10 carbons

in an amount effective to induce differentiation of said cell.

2. The method of claim 1 wherein the number of carbons in R_1 and R_4 taken together is from about 10 to about 28.

3. The method of claim 1 wherein R_1 is C_1 to C_{20} alkyl or C_1 to C_{12} alkyl or alkenyl.

4. The method of claim 2 wherein the number of carbons in R_1 and R_4 taken together is from about 12 to about 26.

5. The method of claim 2 wherein the number of carbons in R_1 and R_4 taken together is from about 14 to about 24.

6. The method of claim 1 wherein R_1 is C_{15} alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5, and R_5 is methyl.

7. The method of claim 1 wherein R_1 is C_{15} alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5, and R_5 is pentanyl.

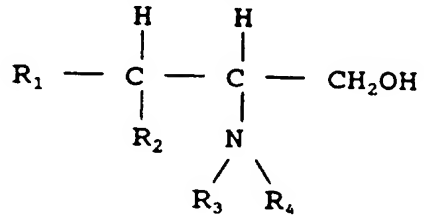
8. The method of claim 1 wherein R_1 is C_8 alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5, and R_5 is heptanyl.

9. The method of claim 1 wherein R_1 is C_{15} alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5, and R_5 is C_{17} alkyl.

10. The method of claim 1 wherein R_1 is C_{15} alkenyl, R_2 is methoxy, R_3 is H, R_4 is COR_5 and R_5 is methyl.

11. The method of claim 1 wherein said cell is a leukemic lymphocyte.

12. A pharmaceutical preparation for inducing cellular differentiation comprising a pharmaceutically acceptable carrier and a compound having the formula



wherein R_1 is C_1 to C_{20} or C_1 to C_{12} alkyl or alkenyl;

R_2 is hydroxyl, alkoxy or H

R_3 is H or lower alkyl;

R_4 is COR_5, SO_2R_5, or CSR_5, where R_5 is C_1 to C_{20} alkyl, alkenyl, or alkynyl, which may be substituted by one or more of the following functional groups: OH, SH, OR_6, SR_6, NR_7R_8, $COOR_9$, and $CONR_{10}R_8$, where R_6, R_7, R_8, R_9, and R_{10} independently are H, lower alkyl, aryl, and arylalkyl; and

wherein the number of carbons in R_1 and R_4 taken together is from about 10 to about 28.

13. The composition of claim 12 wherein the number of carbons in R_1 and R_4 taken together is from about 10 to about 28.

14. The preparation of claim 12 wherein R_1 is C_1 to C_{20} or C_1 to C_{12} alkyl or alkenyl.

15. The preparation of claim 13 wherein the number of carbons in R_1 and R_4 taken together is from about 12 to about 26.

16. The composition of claim 13 wherein the number of carbons in R_1 and R_4 taken together is from about 14 to about 24.

17. The composition of claim 12 wherein R_1 is C_{15} alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5, and R_5 is methyl.

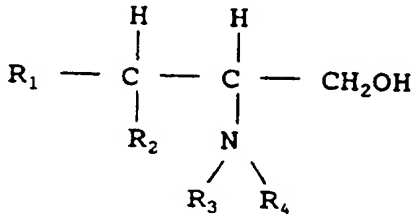
18. The composition of claim 12 wherein R_1 is C_{15} alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5, and R_5 is pentanyl.

19. The composition of claim 12 wherein R_1 is C_8 alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5, and R_5 is heptanyl.

20. The composition of claim 12 wherein R_1 is C_{15} alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5, and R_5 is C_{17} alkyl.

21. The composition of claim 12 wherein R_1 is C_{15} alkenyl, R_2 is methoxy, R_3 is H, R_4 is COR_5, and R_5 is methyl.

22. The use of a compound having the formula



wherein R_1 is C_1 to C_{20} or C_1 to C_{12} alkyl or alkenyl;

R_2 is hydroxyl, alkoxy or H

R_3 is H or lower alkyl;

R_4 is COR_5, SO_2R_5, or CSR_5, where R_5 is C_1 to C_{20} alkyl, alkenyl, or alkynyl, which may be substituted by one or more of the following functional groups: OH, SH, OR_6, SR_6, NR_7R_8, $COOR_9$, and $CONR_{10}R_8$, where R_6, R_7, R_8, R_9, and R_{10} independently are H, lower alkyl, aryl, and arylalkyl; and

wherein the number of carbons in R_1 and R_4 taken together is from about 10 to about 28,

in the preparation or manufacture of a medicament for inducing differentiation of cells.

23. The use of claim 22 wherein said compound is present in the medicament in a therapeutically effective amount.

24. The use of claim 22 wherein the number of carbons in R_1 and R_4 taken together is from about 10 to about 28.

25. The use of claim 22 wherein R_1 is C_1 to C_{20} alkyl or C_1 to C_{12} alkyl or alkenyl.

26. The use of claim 24 wherein the number of carbons in R_1 and R_4 taken together is from about 12 to about 26.

27. The use of claim 24 wherein the number of carbons in R_1 and R_4 taken together is from about 14 to about 24.

28. The use of claim 22 wherein R_1 is C_{15} alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5, and R_5 is methyl.

29. The use of claim 22 wherein R_1 is C_{15} alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5, and R_5 is pentanyl.

30. The use of claim 22 wherein R_1 is C_8 alkenyl, R_2 is

hydroxyl, R_3 is H, R_4 is COR_5, and R_5 is heptanyl.

31. The use of claim 22 wherein R_1 is C_{15} alkenyl, R_2 is hydroxyl, R_3 is H, R_4 is COR_5, and R_5 is C_{17} alkyl.

32. The use of claim 22 wherein R_1 is C_{15} alkenyl, R_2 is methoxy, R_3 is H, R_4 is COR_5 and R_5 is methyl.

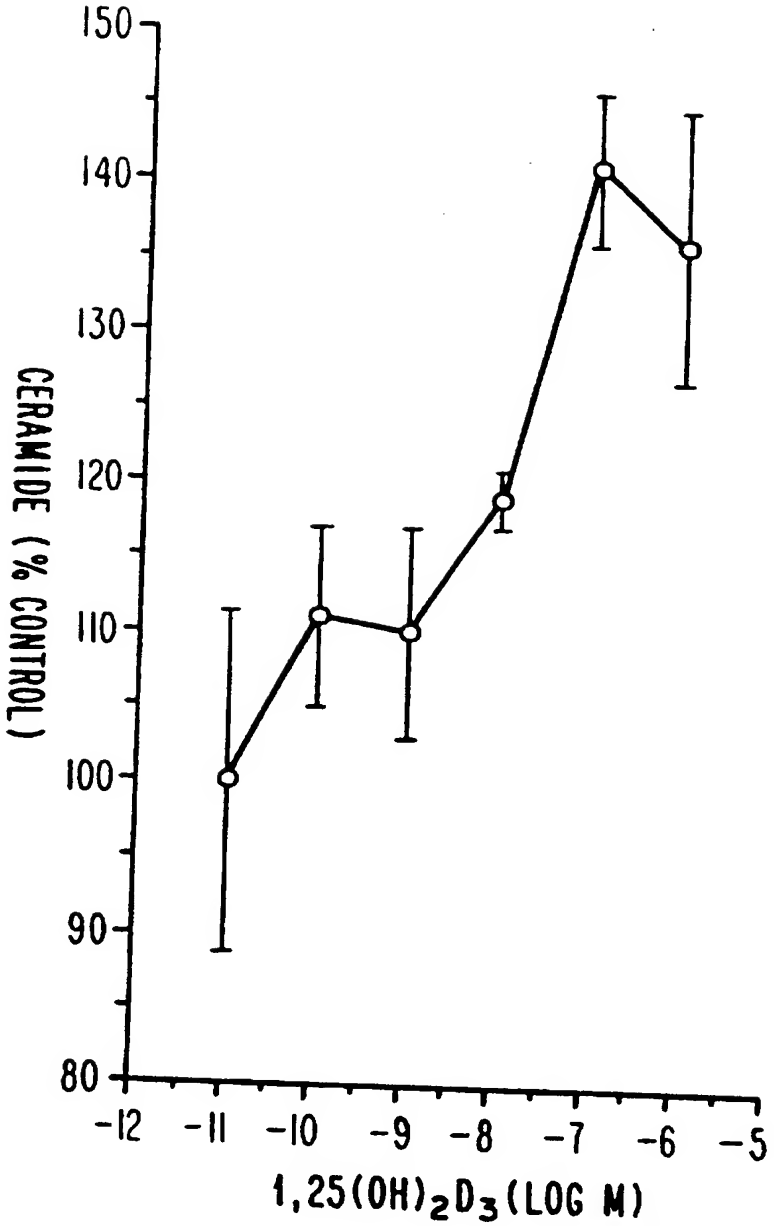


Fig. 1

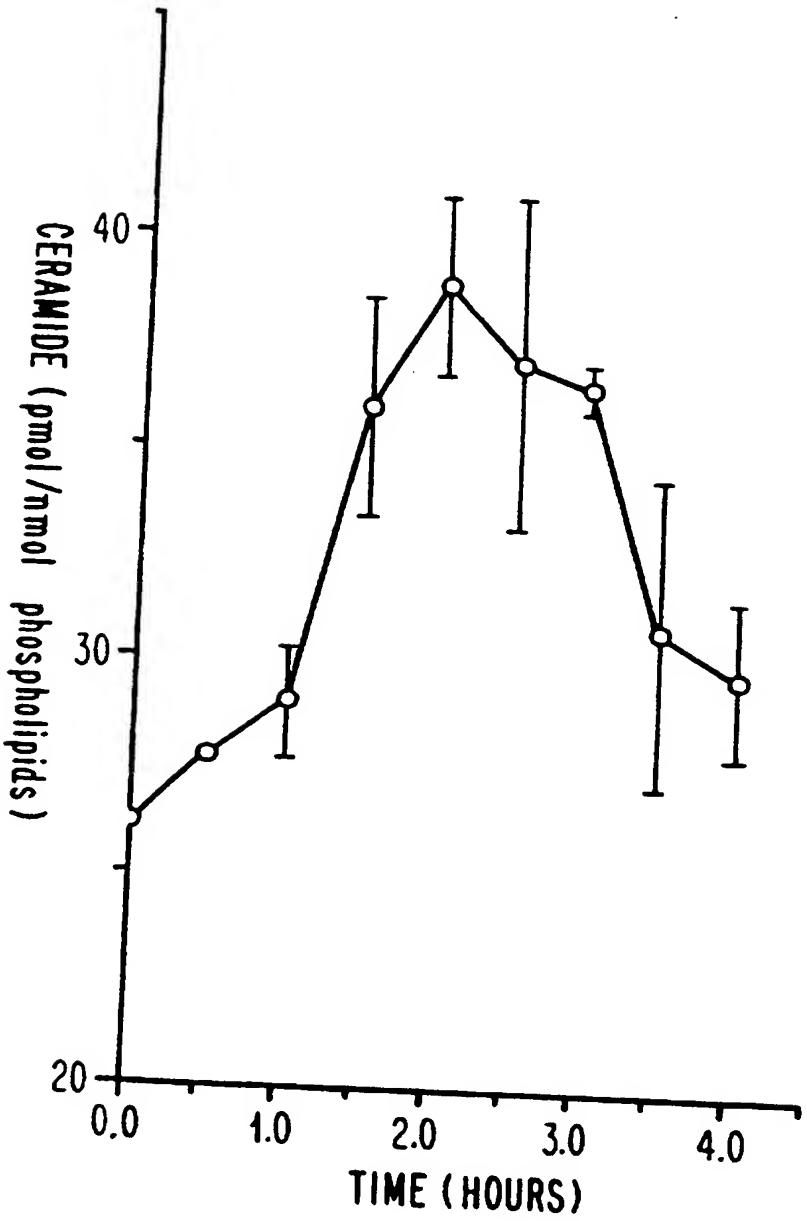

CERAMIDE (pmol/nmol phospholipids)
40
30
20
0.0
1.0
2.0
3.0
4.0
TIME (HOURS)

Fig. 2

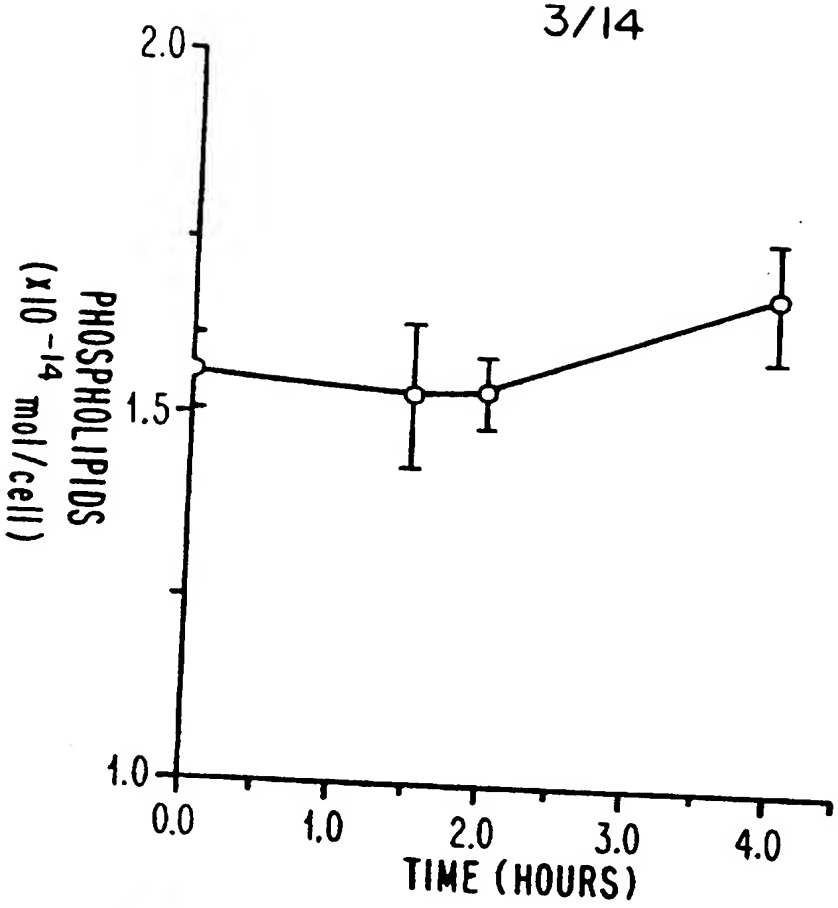


Fig. 3A

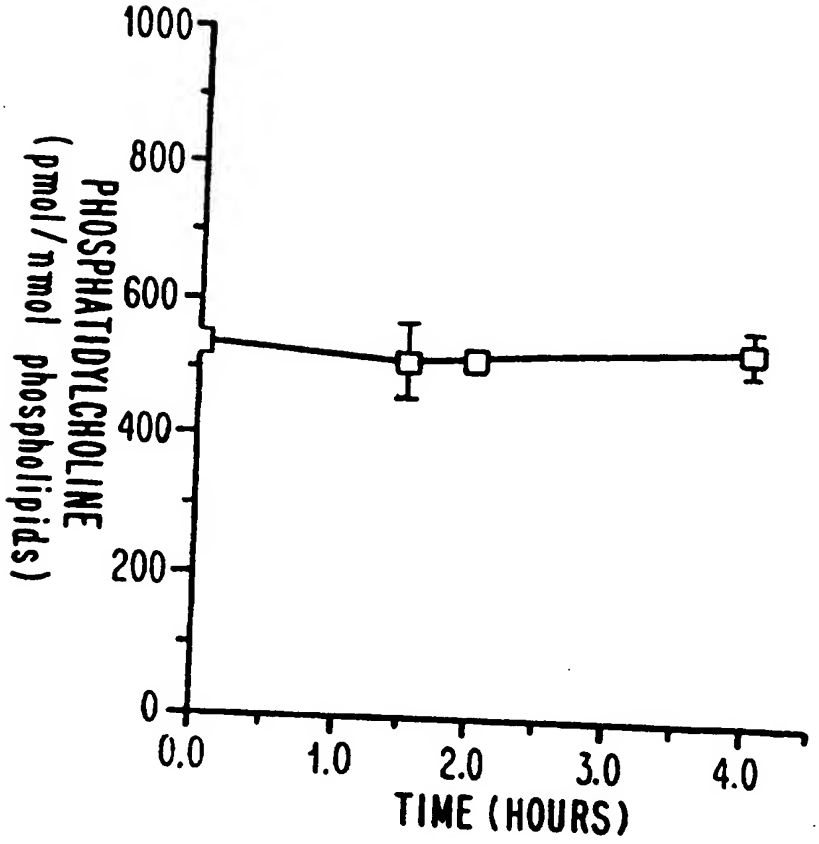


Fig. 3B

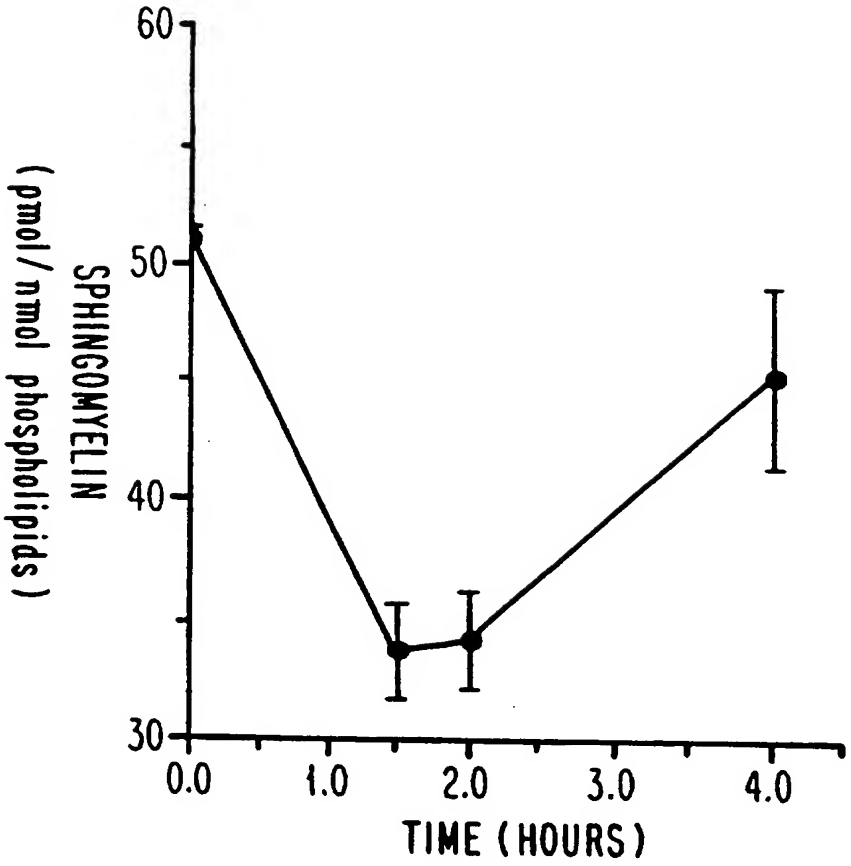

60
50
40
30
0.0
1.0
2.0
3.0
4.0
TIME (HOURS)
SPHINGOMYELIN
(pmol/nmol phospholipids)

Fig. 3C

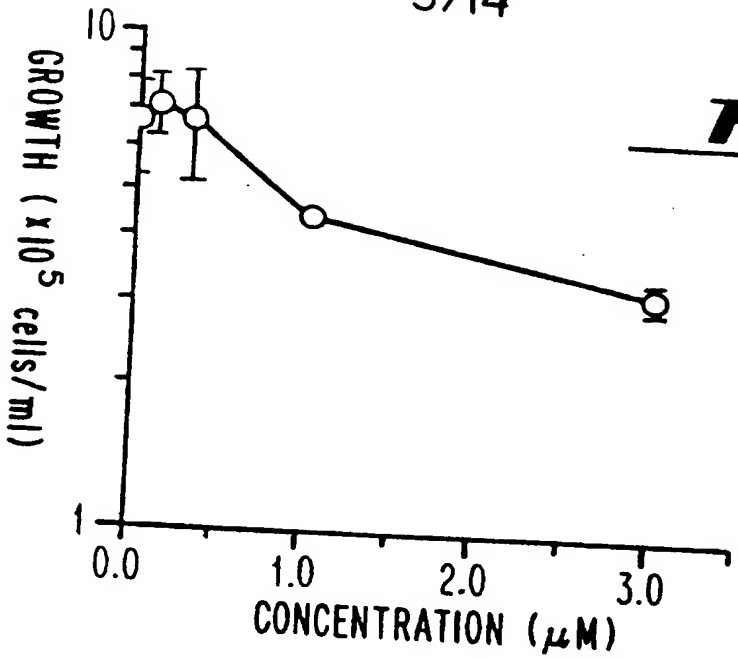

GROWTH (x10^5 cells/ml)
10
1
0.0
1.0
2.0
3.0
CONCENTRATION (μM)

Fig. 4B

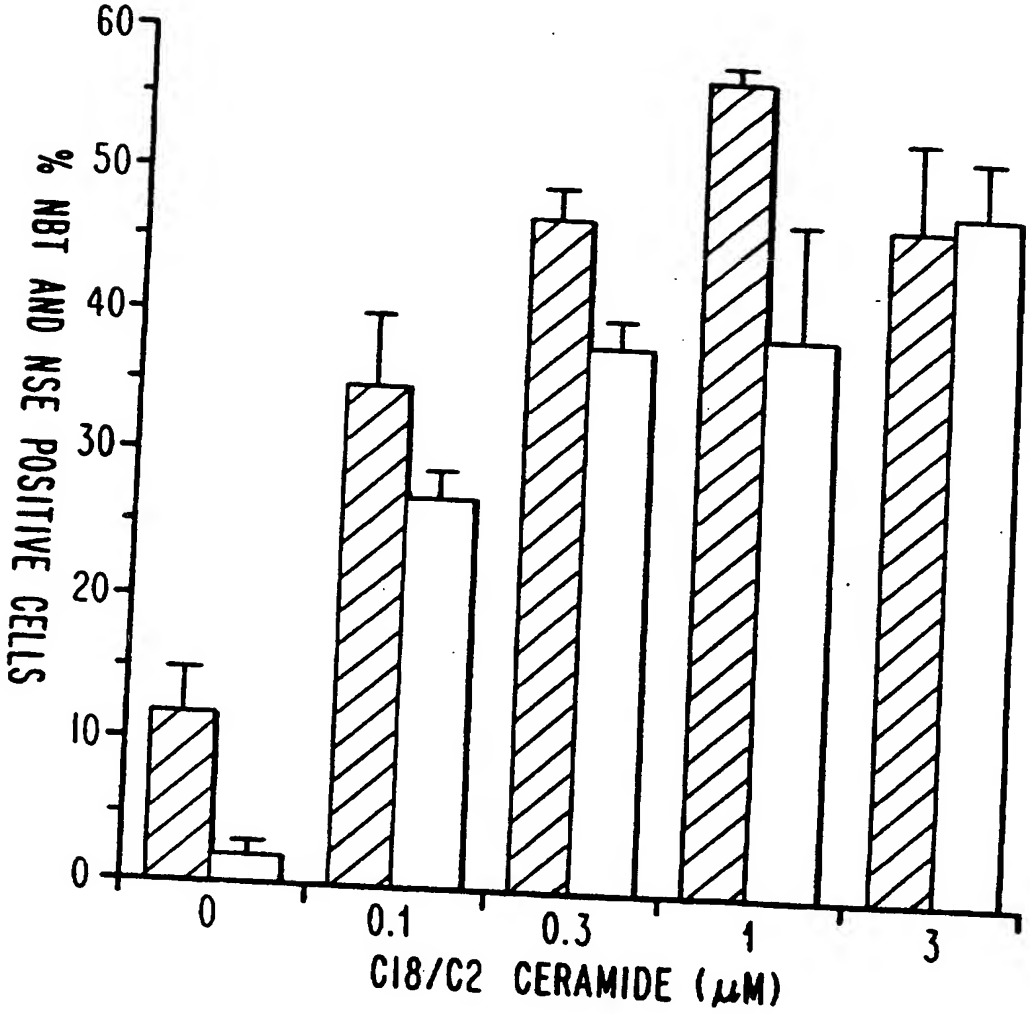

% NBT AND NSE POSITIVE CELLS
60
50
40
30
20
10
0
0
0.1
0.3
1
3
C18/C2 CERAMIDE (μM)

Fig. 4A

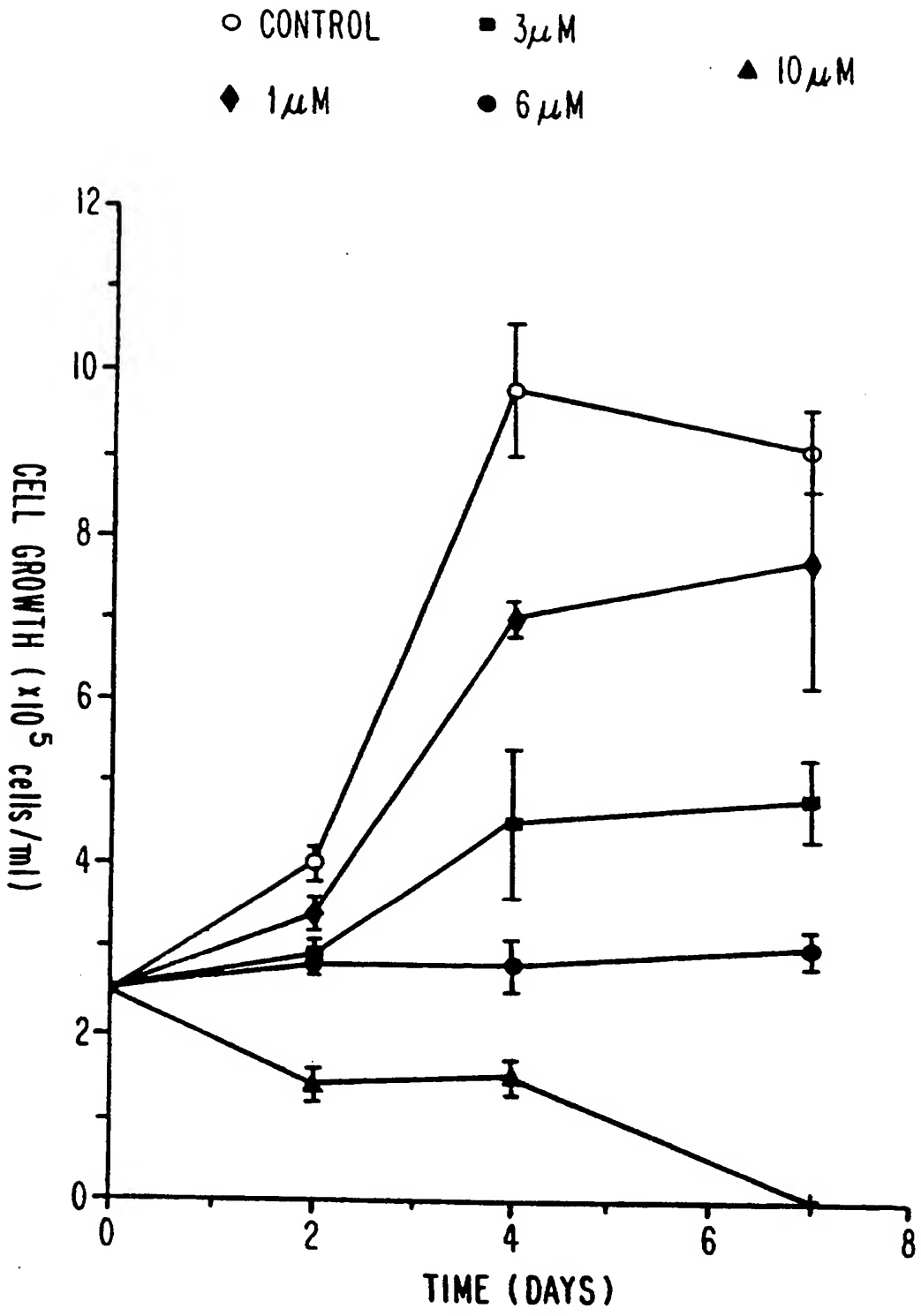

○ CONTROL
■ 3μM
▲ 10μM
◆ 1μM
● 6μM
CELL GROWTH (x10⁵ cells/ml)
TIME (DAYS)
0
2
4
6
8
10
12

Fig. 5

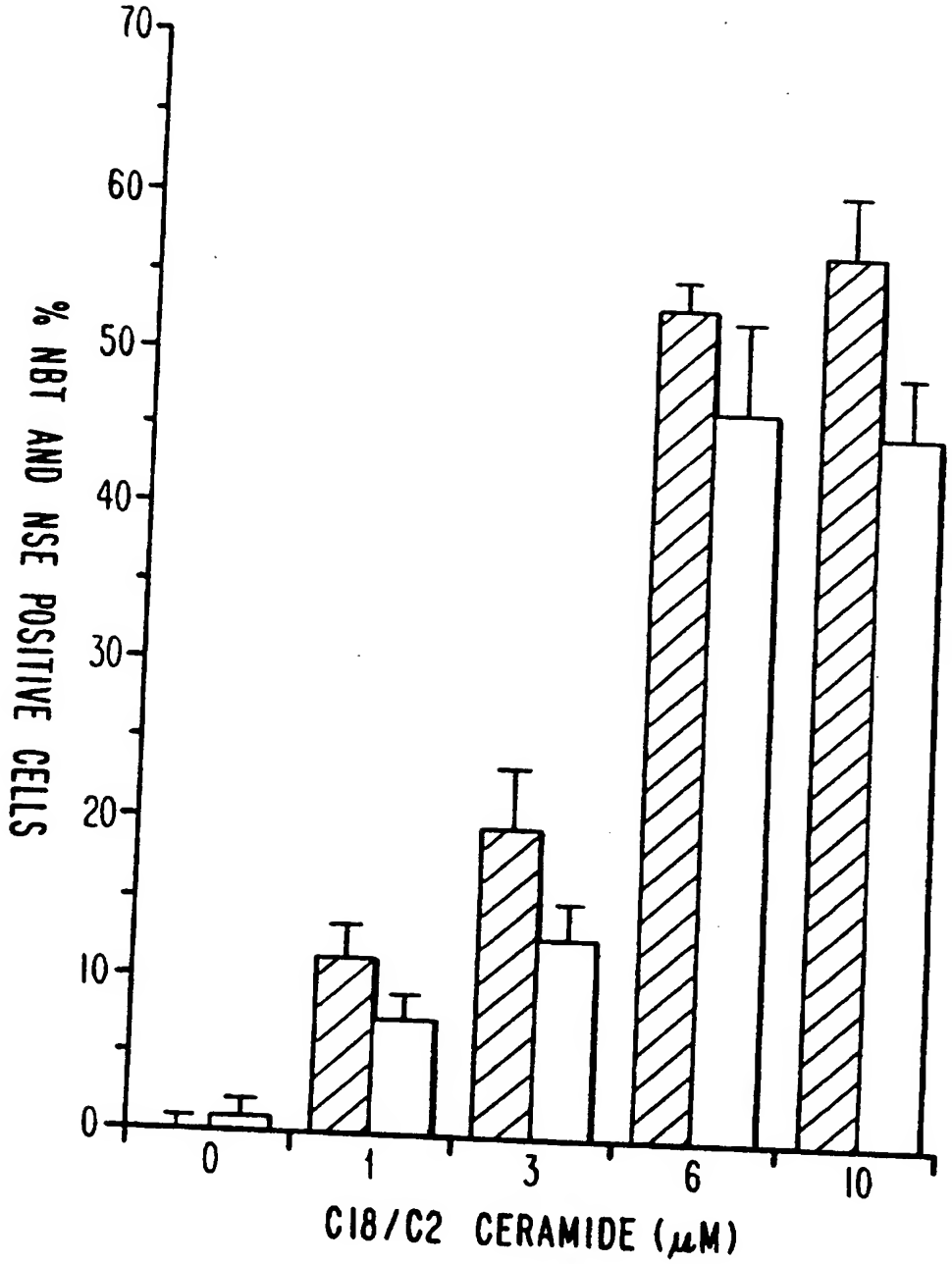

70
60
50
40
30
20
10
0
% NBT AND NSE POSITIVE CELLS
0
1
3
6
10
C18/C2 CERAMIDE (μM)

Fig. 6

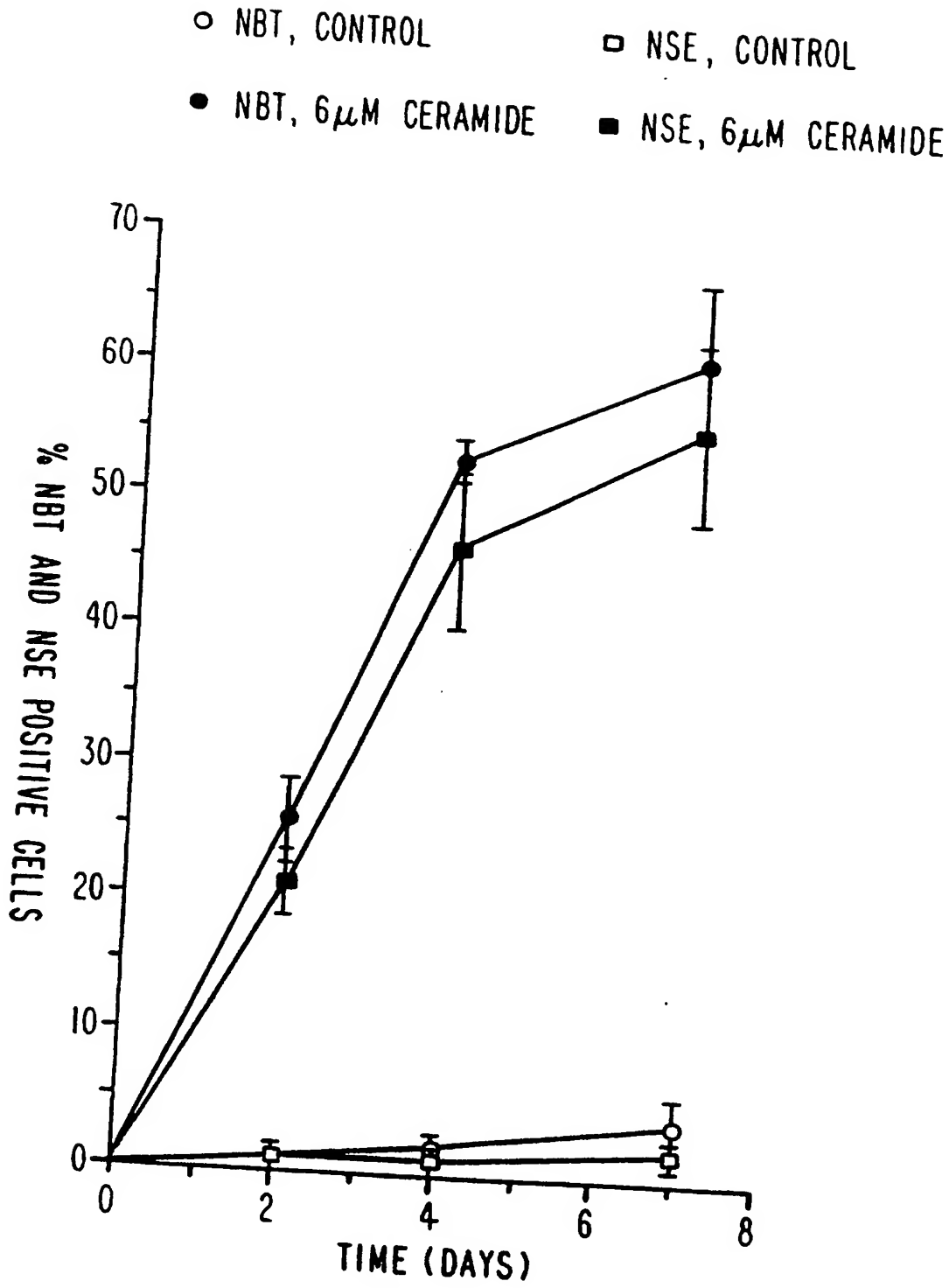

NBT, CONTROL
NSE, CONTROL
NBT, 6μM CERAMIDE
NSE, 6μM CERAMIDE
% NBT AND NSE POSITIVE CELLS
TIME (DAYS)
0
10
20
30
40
50
60
70
0
2
4
6
8

Fig. 7

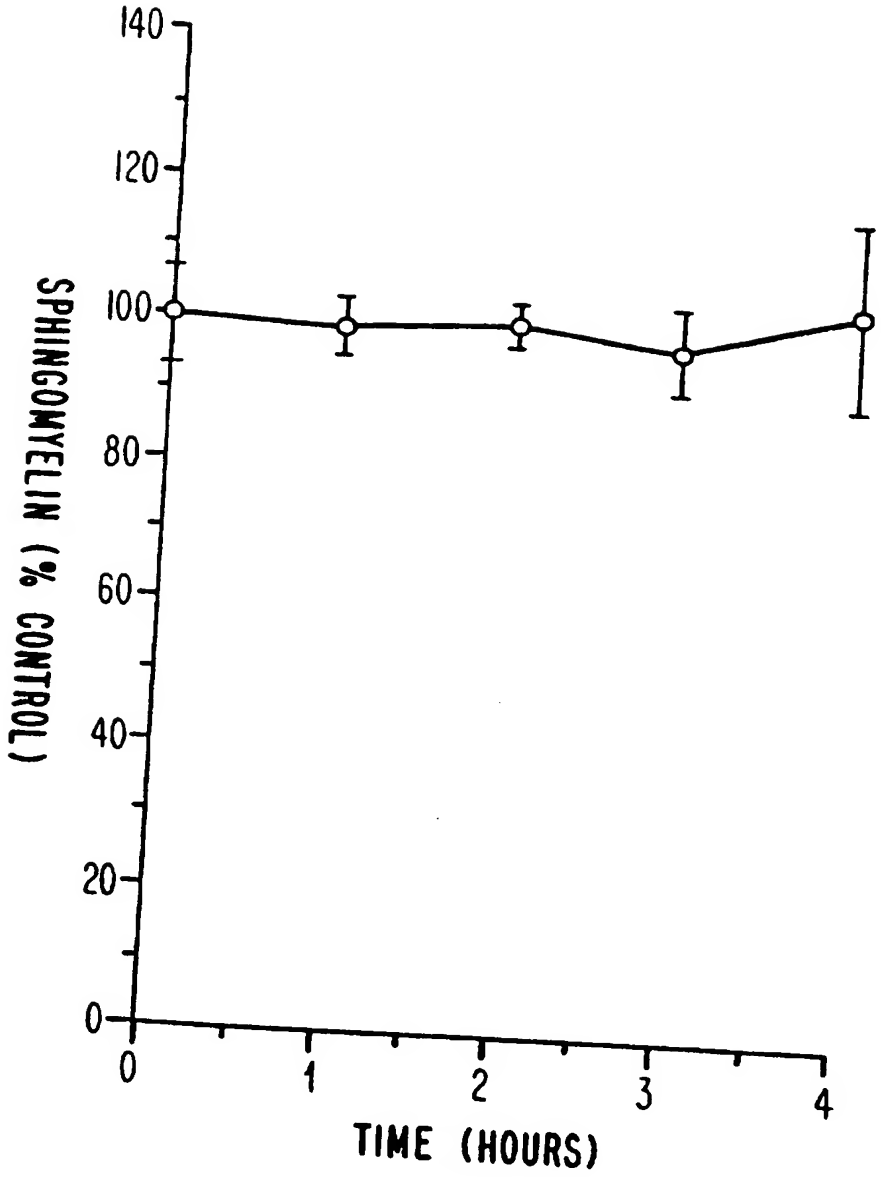

140
120
100
80
60
40
20
0
SPHINGOMYELIN (% CONTROL)
0
1
2
3
4
TIME (HOURS)

Fig. 8

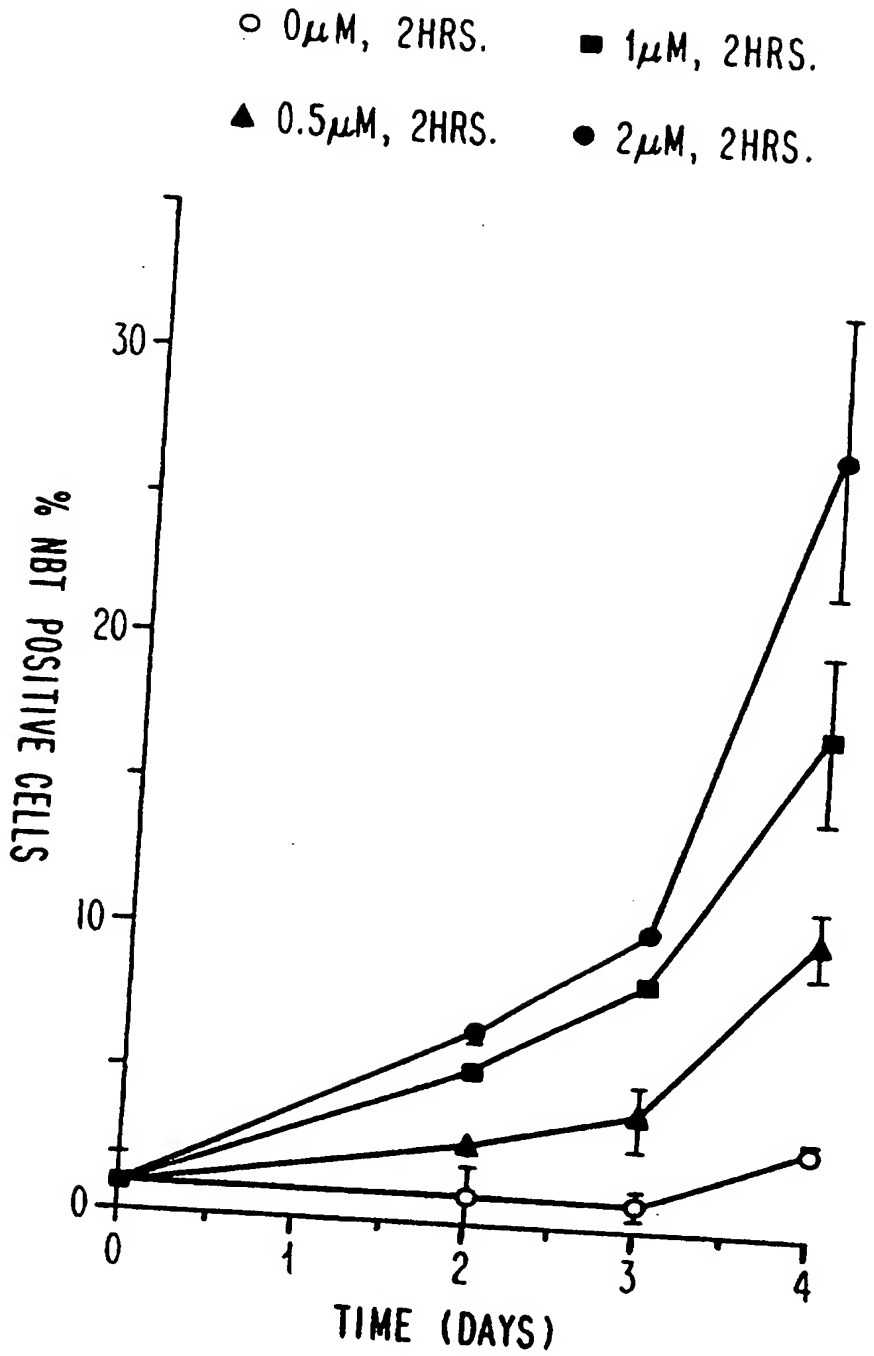

○ 0μM, 2HRS.
■ 1μM, 2HRS.
▲ 0.5μM, 2HRS.
● 2μM, 2HRS.
% NBT POSITIVE CELLS
30
20
10
0
0
1
2
3
4
TIME (DAYS)

Fig. 9

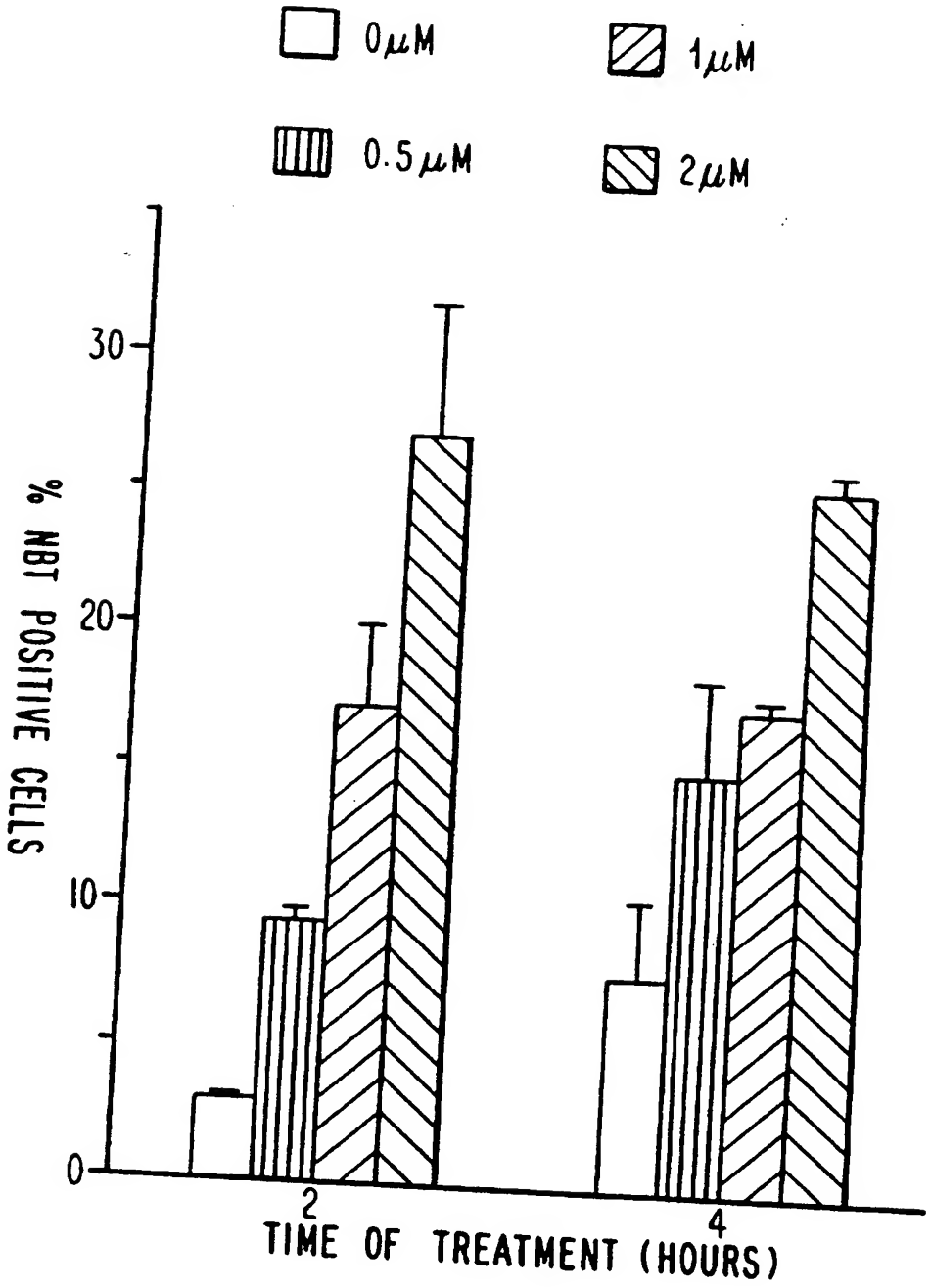

0μM
1μM
0.5μM
2μM
% NBT POSITIVE CELLS
30
20
10
0
2
4
TIME OF TREATMENT (HOURS)

Fig. 10

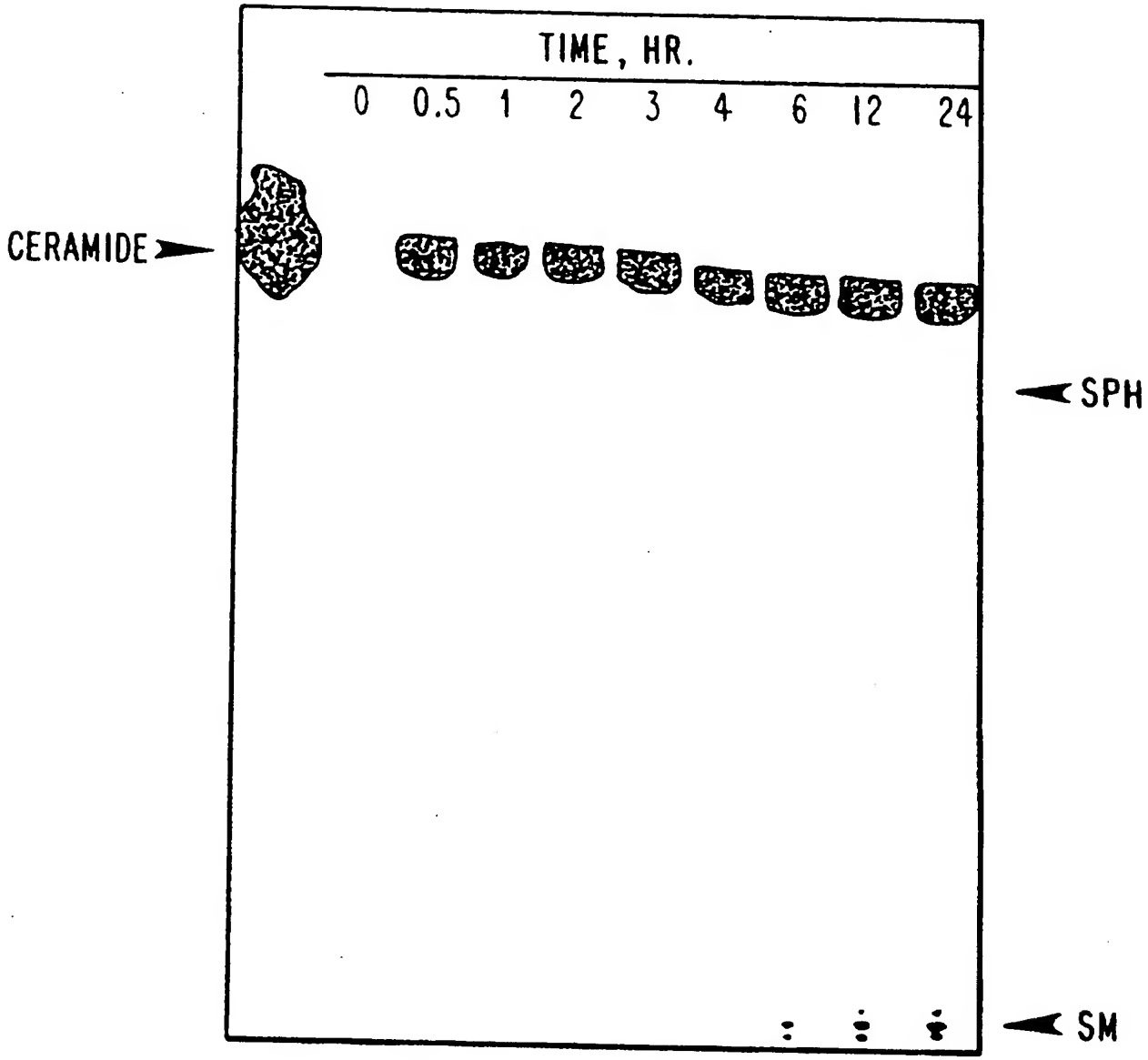


Fig. 11

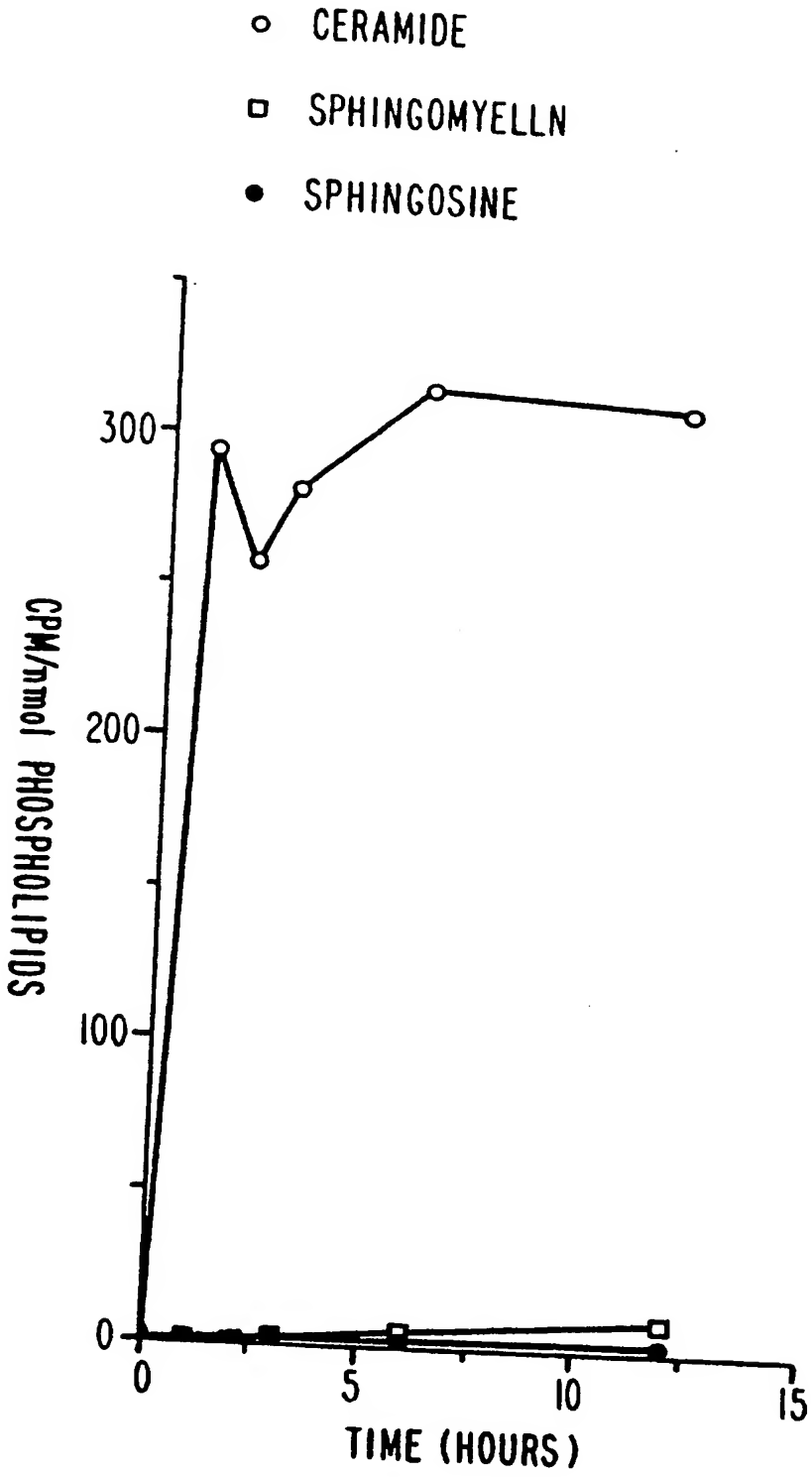

CERAMIDE
SPHINGOMYELLN
SPHINGOSINE
CPM/nmol PHOSPHOLIPIDS
300
200
100
0
0
5
10
15
TIME (HOURS)

Fig. 12

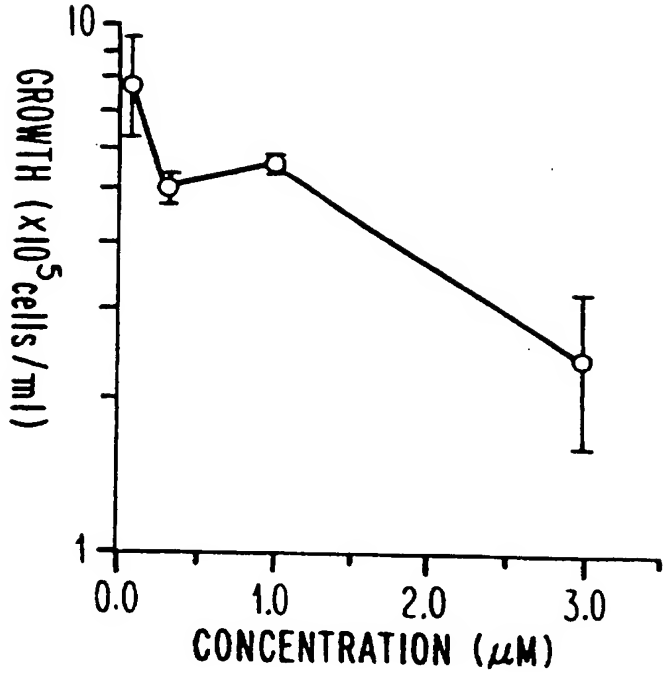


Fig. 13B

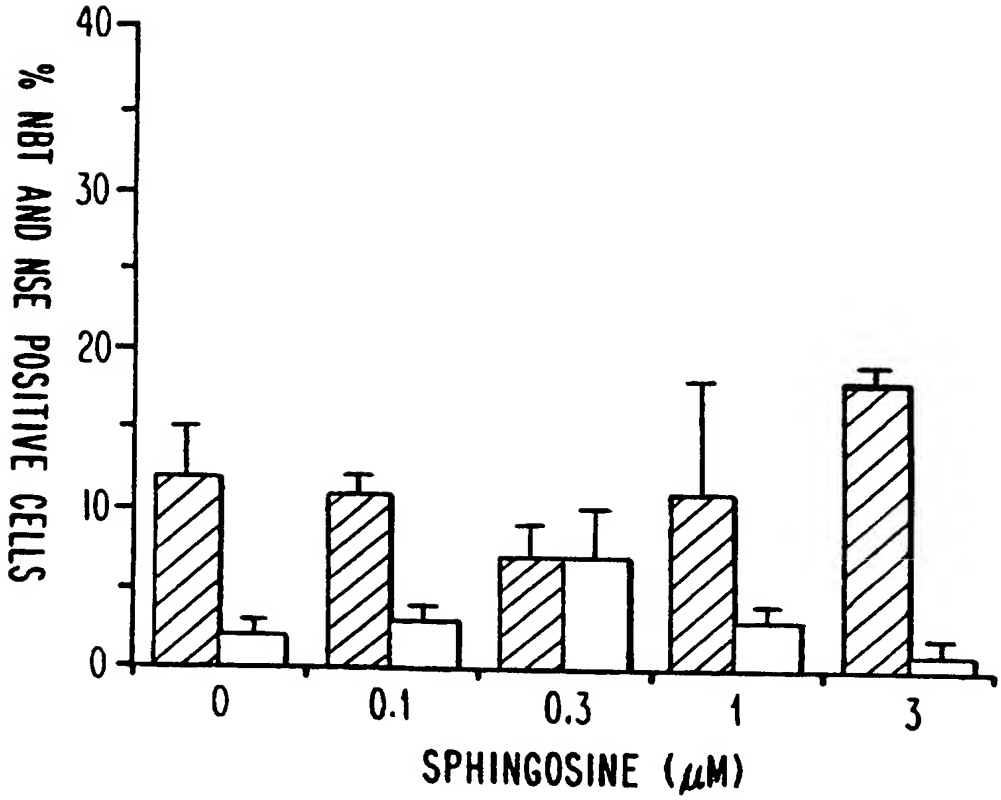


Fig. 13A

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/05743

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 31/13
U.S.Cl.: 435/240.2; 514/625, 629

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl.:	435/240.2; 514/625,629

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS, CAS structures

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Agricultural Biological Chemistry, volume 50, issued 1986, FUNAKI et al., "Synthesis and Biological Activity of the Isomers and Analogs of CHE, 8E,2S, 3R,2'R)-N-2'-Hydroxyhexadecanoyl-9-methyl-4,8-spingacienine, the ceramide portion of the Fruitins-inducing Cerebnoside in a Basidimycete Schizophyllum commune, pages 615-623, see abstract.	1.12.22 1-32
X Y	Journal of Lipid Research, volume 29, issued 1988, Ponec et al., "Lipid composition of cultured human Keratinocytes in relation to their differentiation, pages 949-961, see abstract.	1.12.22 1-32
X	Biochemistry, volume 28, issued 1989, Merrill et al., "Structural Requirements for Long Chain (Sphingoid) Base Inhibitive of Protein Kenase C in vitro and or the Cellular Effects of These Compounds", pages 3138-3145, see table 1.	12-17, 22-28

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 October 1991	18 DEC 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	S. Saucier

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Biochemica et Biophysica Acta, volume 573, issued 1979. Vunnam et al.. "Short chain ceramides as substrates for glucocerebroside synthetase: pages 73-82. see table 1.	12,16,19-20,22-27
X	JP,A, 01093562 (Sugimoto et al.) 12 April 1989. see entire document.	1-9,11-20, 22-31
X Y	Journal of Biological Chemistry, volume 264. No. 32. issued 15 November 1989. Okazaki et al., "Sphingomychin Turnover Induced by Vitamin D_3 in HL-60 cells", pages 19076-19080, see Fig. 2.	1,12,22 1-32
X Y	Journal of Biological Chemistry, volume 265, No. 7, issued 05 March 1990, Lavie et al.. "Activation of Phospholipase D by Spingord Bases in NG108-15 Neural-derived cells". pages 3868-3872. see table 1.	1,12,22 1-32
X Y	Biochemica et Biophysica Acta. volume 1021, issued 1990, Abraham et al, "Interactin between corneocytes and shatum corneum lipid liposomes in vitro, pages 119-125. see abstract.	1,12,22 1-32
X.P Y	Journal of Investigative Dermatology Inc.. volume 95. No. 6. issued December 1990. Madison et al., "Sphingolipid Metabolism in Organotypic Mouse Keratinocyte Cultures", pages 657-664, see abstract.	1,12,22 1-32
X Y	Clinical Research. volume 38. No. 2. issued 4-7 May 1990, Okazaki et al., "Function of Ceramide a Lipid Mediator of Differentiation in HL-60 cells" page 367A. see entire article.	1,12,22 1-32
X Y	Tetraheron Letters, volume 30, No. 51, issued 1989, Shibuya et al., "Synthesis of two Pairs of Enantiomeric C_{18}-Spingosines" pages 7205-7208, see page 7207.	12-17, 22-28 12-32
X Y	Analytical Biochemistry, volume 183, issued 1989, Veldhoven et al., "Enzymatic Quantification of Spingosine in the Picomole Range in Cultured Cells", pages 177-189, see figure 1.	12-18, 22-29 12-32

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	Journal of the American Chemical Society, volume 88, issued 05 August 1966, Gaver et al., "Chemistry and Metabolism of Spingolipids. 3-Oko Derivatives of N-Acetylspingosine and N-Acetyldihydrospigosine", pages 3643-3647, see pages 3646-3647.	12-32
Y	Tetrahedron Letters, volume 29, No. 25, issued 1988, Nimkar et al., "A stereoselective Synthesis of Spingosine, A Protein Kinase C Inhibitor", pages 3037-3040, see entire article.	12-32
X, P Y	Journal of Biological Chemistry, volume 265, No. 26, issued 15 September 1990, Okazaki et al., "Role of Ceramide as a Lipid Mediator of 1 , 25-Dihydroxyvortamin D_3-induced HL-60 cell Differentiation", pages 15823-15831, see figure 5.	1-6,12-17,22-28 / 1-32
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